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Tumour evolution over time, treatment and progression; exploring the molecular heterogeneity of oestrogen receptor positive breast cancer

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A thesis submitted in fulfilment of the requirements for the degree of Doctor of Medicine, to the University of Edinburgh, 2017

Declaration

I declare that I am the author of this thesis; that the work herein was completed by myself unless otherwise indicated where the contributor is acknowledged. This work has not been submitted for any other degree.

Laura M Arthur, 2017

Abstract

Introduction

Recent advances in microarray technology have allowed more understanding of the complex molecular biology of breast cancer. The traditional prognostic information afforded by hormone receptor status and pathology variables is being supplemented and superseded by gene signatures predictive of risk of recurrence and response to treatments. Approximately 75% of breast cancers are oestrogen receptor positive (ER+) and can be treated by drugs that block oestrogen production such as letrozole. However not all ER+ tumours respond and even those that initially respond can develop resistance.

Treating patients with neoadjuvant letrozole affords a unique opportunity to sample the same tumour in vivo at different time points reducing any potential inter-patient and inter-tumour variability. The molecular effects of drugs can be assessed long before clinical outcome is apparent. Underlying genetic differences or characteristics of the patient, tumour or sample may affect the molecular response to treatment.

This project set out to use sequential patient-matched samples to evaluate molecular changes in breast tumours in the presence or absence of endocrine treatment in different subtypes, defined by histology or mutation status and to assess molecular variation between primary tumour and nodal metastasis.

Methods

RNA was extracted and processed to generate whole transcriptome Illumina Beadarray gene expression data from four unique cohorts of patients. Clinical data on treatments, recurrence and survival was collected from medical records.

The first cohort compared 25 breast cancer patients with matched samples at diagnosis and at surgery, 14-35 (median 23) days later, with no intervening treatment; with 36 patients treated with neoadjuvant letrozole.

A PCR assay to detect 8 known *PIK3CA* mutations and assessment of PTEN status was performed at both the primary and secondary event in a second cohort of 120 patients with endocrine treated disease who relapsed with either recurrence, lymph node metastases, a new second primary or progression of disease on primary endocrine therapy.

The third cohort compared the molecular response to neoadjuvant letrozole in 14 patients with invasive lobular cancer (ILC) and 14 patients with invasive ductal cancer (IDC).

A fourth cohort of women with node positive disease at diagnosis were assessed for variations in gene expression profiles between primary tumour and synchronous metastatic axillary lymph nodes (68 samples from 31 patients).

Results

The genomic profile of the no intervening treatment cohort did not differ significantly. Some changes in inflammatory genes were evident. This reassures us that changes seen during treatment are truly due to drug effect. This validates the use of a second biopsy to explore prediction of response.

PIK3CA mutation status is maintained in the majority of patients with endocrine resistant disease and changed in only 15.7%. Where there was a change in *PIK3CA* this was significantly more likely to be a second primary breast cancer rather than a recurrence or progression of the primary cancer. PTEN status was also maintained in most patients. This does not support the theory that acquisition of a *PIK3CA* mutation is responsible for developing endocrine resistance. Novel PI3K inhibitor drugs may still be suitable in endocrine-resistant disease if activation of the pathway develops by other mechanisms.

Consistent with previous studies, significant molecular differences were observed between ILC and IDC pre-treatment. Over half of these molecular differences were maintained after 3 months of letrozole. However, changes over time in individual tumours in response to letrozole were highly consistent in both ILC and IDC.

When comparing primary with synchronous metastatic nodes only 39% of tumours clustered together with their matched primary or node. The molecular subtype of the node was often a poorer prognosis than the primary. There were also differences in subtype between nodes in a small cohort of patients with 2 involved nodes.

Conclusions

We have demonstrated that neoadjuvant window studies are a valid model for assessment of drug effects and evaluated differences in histology and mutation status.

Endocrine resistance in breast cancer is rarely related to acquisition of *PIK3CA* mutations.

Synchronous lymph node metastases can differ greatly from their matched primary. These findings are highly relevant when considering prescribing (neo)/adjuvant therapy and have significantly improved our understanding of breast cancer as we strive towards personalised medicine.

Lay Summary

Introduction

Recent technological advances have allowed researchers to measure the level of large numbers of genes simultaneously. We can identify which genes are active in cancer and how the level of these genes changes with effects of treatment and time. In breast cancer, this has given rise to numerous 'predictive gene signatures,' where measuring the level of a small number of genes can give information about risk of disease progression and can identify patients suitable for certain specific treatments. This is now used to supplement the traditional staging information given by features of the cells of the tumour assessed by a pathologist using a microscope.

75% of breast cancers possess the oestrogen receptor and these tumours can be treated with anti-oestrogen drugs, such as letrozole. However not all of these tumours respond and some who do respond initially then relapse later. Understanding the mechanism by which tumours achieve this is crucial in advancing treatment.

This project explores the genetic profiles of serial samples taken from the same patient and cancer at different periods in time, treatment and different sites of disease, in order to assess changes in the molecular profiles of the tumour at these points.

Methods

Measurements of gene levels were made from samples of tumours collected from patients before and during treatment. Information on treatments, recurrence and survival was collected from patient medical records. 4 unique cohorts of patients were included.

The first cohort compared 25 breast cancer patients with matched samples at diagnosis and at surgery, 14-35 (median 23) days later, with no treatment in the intervening period; with 36 patients treated with pre-operative letrozole.

The second cohort included 120 patients with endocrine-treated disease who relapsed with either recurrence, lymph node metastases, a new second primary, or progression of disease on primary endocrine therapy. A method to detect 8 known genetic mutations of a cancer pathway gene – *PIK3CA*, was performed on the tumour at both primary and secondary event.

The third cohort compared the gene changes during treatment with letrozole in two groups of 14 patients with the 2 most common subtypes of breast cancer, known as 'lobular' and 'ductal.'

A fourth cohort of women with evidence of cancer spread from the breast to their lymph nodes at diagnosis were assessed for variations in gene expression profiles between the breast tumour and the lymph node tumour (68 samples from 31 patients).

Results

The gene profiles of the tumours with no intervening treatment do not differ significantly. Some changes in inflammatory genes were evident. This reassures us that changes seen during treatment are truly due to drug effect. This validates the use of a second biopsy to explore prediction of response.

PIK3CA mutation status is maintained in the majority of patients with endocrine resistant disease and changed in only 15.7%. Where there was a change in *PIK3CA* this was significantly more likely to be a second primary breast cancer rather than a recurrence or progression of the primary cancer. This does not support the theory that acquisition of a *PIK3CA* mutation is responsible for developing endocrine resistance. Novel drugs inhibiting this cancer pathway may still be suitable in endocrine resistant disease if activation of the pathway develops by other mechanisms.

Consistent with previous studies, significant differences in levels of genes were observed between the lobular and ductal types of breast cancer pre-treatment. Over half of these molecular differences were maintained after 3 months of treatment with letrozole. However, changes over time in individual tumours were highly consistent in both subtypes of breast cancer.

When comparing breast primary tumours with lymph node tumours only 39% of cases clustered together with their matched primary or node. The subtype of the node based upon levels of genes was often a poorer prognosis than the primary. There were also differences in subtype between nodes in a small cohort of patients with 2 involved nodes.

Conclusions

We have demonstrated that pre-operative window studies are a valid model for assessment of drug effects and evaluated differences in histology and mutation status.

Endocrine resistance in breast cancer is rarely related to acquisition of *PIK3CA* mutations.

Lymph node metastases can differ greatly from their matched primary. These findings are highly relevant when considering prescribing targeted treatments and have significantly improved our understanding of breast cancer as we strive towards personalised medicine.

Thesis Organisation and Structure

The results chapters (2-5) within this thesis are presented in the style of international peer-reviewed journal articles. Chapters 3 and 4 have been published as presented in the thesis and Chapter 2 was presented at San Antonio Breast Cancer Symposium 13th December 2013 and published after expanding with additional samples and further analysis. Chapter 5 was presented as a poster at San Antonio Breast Cancer Symposium 7th December 2016. The Figure and Table numbers have been modified to make them more coherent in the context of the overall thesis. A single unified reference list has been created. It is hoped that the result is a clear and concise thesis summarising the valuable contributions of this doctoral project. Brief descriptions of the contributions of others to these chapters and the details of the publications are given below.

Chapter 2 – 18 paired RNA samples (9 patients) were extracted by Dr Vicky Sabine from a previous study funded by GlaxoSmithKline. RNA from the other 32 samples was extracted by myself. All RNA was further processed for microarray by myself. The letrozole treated cohort (n=72 samples, 36 patients) used as comparison was constructed by Dr Arran Turnbull from work in his PhD.

Pearce DA, **Arthur LM**, Turnbull AK, Renshaw L, Sabine VS, Thomas JS, Bartlett JM, Dixon JM, Sims AH. (2016) Tumour sampling method can significantly influence gene expression profiles derived from neoadjuvant window studies *Sci Rep.* 6:29434. PMID: 27384960

Chapter 3 – DNA extraction and *PIK3CA* mutation PCR assay performed by Genentech, San Francisco. PTEN IHC performed by Histogenix, Belgium.

Arthur LM, Turnbull AK, Renshaw L, Keys J, Thomas JS, Wilson TR, Lackner MR, Sims AH, Dixon JM. (2014) Changes in *PIK3CA* mutation status are not associated with recurrence, metastatic disease or progression in endocrine-treated breast cancer. *Breast Cancer Res Treat.* 147(1):211-9. PMID: 25104442

Chapter 4 – Raw data composed by Dr Arran Turnbull previously. I acknowledge and am very thankful for the work in advanced bioinformatics analysis by Dr Andy Sims.

Arthur LM, Turnbull AK, Webber VL, Larionov AA, Renshaw L, Kay C, Thomas JS, Dixon JM, Sims AH. (2014) Molecular changes in lobular breast cancers in response to endocrine therapy. *Cancer Res.* 74(19):5371-6. PMID: 25100562

Chapters 2 and 4 - The letrozole treated cohorts in these chapters were part of a larger cohort constructed and analysed previously by Dr Arran Turnbull, used to develop a 4-gene model predictive of response to neoadjuvant letrozole. I contributed to the following publication in assisting with development of the PCR and IHC techniques to implement the predictive model. Turnbull AK, **Arthur LM**, Renshaw L, Larionov AA, Kay C, Dunbier AK, Thomas JS, Dowsett M, Sims AH, Dixon JM. (2015) Accurate prediction and validation of response to endocrine therapy in breast cancer. *J Clin Oncol.* 33(20):2270-8. PMID: 26033813

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And sorry for the stress!

List of figures

List of tables

Table of Contents

Abbreviations

ABC	Advanced Breast Cancer consensus group
ACIS	Automated cellular imaging system
ACOSOG	American College of Surgeons Oncology Group
Agr2	Anterior gradient protein 2
AI	Aromatase inhibitor
ALCAM	Activated leukocyte cell adhesion molecule
ALH	Atypical lobular hyperplasia
ALTERNATE	ALTernate approaches for clinical stage II or III Estrogen Receptor positive breast cancer NeoAdjuvant TrEatment
ANOVA	Analysis of variance
AO	Adjuvant! Online
AOC3	Amine oxidase, copper containing 3
AP-1	Activating protein 1
AQUA	Automated quantitative analysis
AR	Androgen receptor
ASCO	American Society for Clinical Oncology
ASPM	Abnormal spindle microtubule assembly
ATF-3	Activating transcription factor 3
ATLAS	Adjuvant Tamoxifen: Longer Against Shorter
BCI	Breast cancer index
BELLE-2	Buparlisib helps overcome endocrine resistance in metastatic breast cancer
BIG	Breast International Group
BL	Baseline
BLAST	Basic Local Alignment Search Tool
BOLERO-2	Breast Cancer Trials of Oral Everolimus-2
BRCA	Breast and ovarian cancer susceptibility protein
BRF2	B related factor 2
BUB1	Budding uninhibited by benzimidazoles
CA12	Carbonic anhydrase 12
CAV1	Caveolin 1
CCNB1	Cyclin B1
CCND1	Cyclin D1
CDC2	Cell division control protein 2 homolog
CDH1	E-Cadherin encoding gene
CDK	Cyclin dependent kinases
cDNA	Complementary DNA
CI	Confidence interval
CK 5/6	Cytokeratin 5/6
CKS2	CDC28 protein kinase regulatory subunit 2
CNAs	Copy number aberrations
COL1A1	Collagen type 1 alpha 1 chain

COLEC12	Collectin subfamily member 12
CONFIRM	Comparison of Faslodex in Recurrent or Metastatic Breast Cancer
CSC	Cancer stem cell
CTC	Circulating tumour cells
ctDNA	Circulating cell-free tumour DNA
CXCL12	C-X-C motif chemokine ligand 12
CXCR4	Chemokine receptor
CYP19	Cytochrome P450 enzyme gene, encodes aromatase
DAVID	Database for Annotation Visualization and Integrated Discovery
DCIS	Ductal carcinoma in situ
DFS	Disease free survival
DNA	Deoxyribonucleic acid
DNMTs	DNA methytransferases
DPT	Dermatopontin
ECM	Extracellular matrix
EGFR	Human epidermal growth factor receptor
EP / EPclin	Endopredict / EndopredictClin
EPCAM	Epithelial cell adhesion molecule
ER / ER+	Oestrogen receptor / Oestrogen receptor alpha positive
ERBB2	Erb-B2 receptor tyrosine kinase 2
ERE	Oestrogen response element
ESM1	Endothelial cell specific molecule 1
ESR1	Gene encoding oestrogen receptor alpha
ET	Endocrine therapy
et al	Et alia
FALCON	Fulvestrant 500mg versus anastrozole 1mg for hormone receptor-positive advanced breast cancer
FDR	False discovery rate
FFPE	Formalin fixed paraffin embedded
FGFR	Fibroblast growth factor receptor
FIRST	Fulvestrant First-Line Study Comparing Endocrine Treatments
FISH	Fluorescence in situ hybridisation
FLT1	Fms related tyrosine kinase 1
FOSB	FosB proto-oncogene
FOXA1	Forkhead box A1
G1-Phase	Gap 1 phase
GATA3	GATA binding protein 3
GEO	Gene expression omnibus
GGI	Genomic Grade Index
GIDE	Global Index of Dependence on oEstrogen
GnRH	Gonadotrophin releasing hormone agonists
GRB7	Growth factor receptor bound proteins
GSEA	Gene set enrichment analysis

H:I ratio	HOXB13:IL7BR ratio
H&E	Haematoxylin and Eosin
HCL	Hierarchical clustering
HDACs	Histone deacetylases
HER2 / 3 / 4	Human epidermal growth factor receptor 2 / 3 / 4
HGF	Hepatocyte growth factor
HNF3	Hepatocyte nuclear factor 3
HR	Hazard ratio
IC	Integrated cluster
ID	Identifier
IDC	Invasive ductal carcinoma
IER2	Immediate early response 2
IGF / R / BP	Insulin-like growth factor / receptor / binding protein
IHC	Immunohistochemistry
IL6ST	Interleukin 6 signal transducer
ILC	Invasive lobular carcinoma
IMPACT	Immediate Preoperative Anastrozole, Tamoxifen, or Combined with Tamoxifen
INPP4B	Inositol polyphosphate-4-phosphatase type II B
ITGB2	Integrin subunit beta 2
LCIS	Lobular carcinoma in situ
LETM2	Leucine zipper and EF hand containing protein
LN	Lobular neoplasia
LREC	Local research ethics committee
MADP2	Mitotic feedback control protein 2 homolog
MAP / K	Mitogen activated protein / kinase
MBD	Methyl-CpG-binding domain
MCM4 / 6	Minichromosome maintenance complex component 4 / 6
MDS	Multidimensional scaling plot
MDT	Multi-disciplinary team
METABRIC	Molecular Taxonomy of Breast Cancer International Consortium
MGI	Molecular grade index
MGPS	MultiGene Proliferation Score
MINDACT	Microarray In node negative and 1-3 positive lymph node disease may avoid chemotherapy
MKLP	Mitotic kinesin-like protein
MMP	Matrix metallopepitadase/metalloproteinase
MONALEESA-2	Mammary oncology assessment of LEE011's (Ribociclib's) Efficacy and Safety
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
MTAP	Methylthioadenosine phosphorylase
mTOR	mammalian Target of Rapamycin
MYBL2	MYB proto-oncogene like 2
MYC	V-Myc avian myelocytomatosis viral oncogene homolog

N	Node
n	Sample size
N-COR	Nuclear receptor corepressor
NEDD9	Neural precursor cell expressed developmentally down-regulated 9
NEOCENT	Neoadjuvant Endocrine vs Chemotherapy Trial
neoMONARCH	A Neoadjuvant Study of Abemaciclib (LY2835219) in Postmenopausal Women with Hormone Receptor Positive, HER2 Negative Breast Cancer
NPI	Nottingham Prognostic Index
NR	Not recorded
NR3C2	Nuclear receptor subfamily 3 group c member 2
OS	Ovarian suppression
P	Primary tumour
p value	probability
PALOMA 2	Palbociclib: Ongoing trials in management of breast cancer
PALOMA-3	Fulvestrant plus palbociclib versus fulvestrant plus placebo for treatment of hormone-receptor-positive, HER2-negative metastatic breast cancer that progressed on previous endocrine therapy
PANTHER	Protein analysis through evolutionary relationships
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
pCR	Pathological complete response
PDE4B	Phosphodiesterase 4B
PDK1	Pyruvate dehydrogenase kinase 1
PELP1	Proline glutamate and leucine riche protein 1
PEPI	Preoperative Endocrine Prognostic Index
PFP	Percent false present
PFS	Progression free survival
PGAP3	Post GP1 attachment proteins
PgR	Progesterone receptor encoding gene
PI3K	Phosphatidylinositol 3-kinase
PIK3CA/B	Phosphatidylinositol-4,5-biphosphate 3-kinase catalytic subunit α/β
PIK3R1	Phosphoinositide-3-kinase regulatory subunit 1
PLIN1	Perilipin 1
POETIC	PeriOperative Endocrine Therapy: Individualizing Care
PPAPDC1B	Phosphatidate phosphatase
PPP2R2A	Protein phosphatase 2 regulatory subunit alpha
PR	Progesterone receptor
PROACT	Preoperative Anastrozole Compared with Tamoxifen
PTEN	Phosphatase and tensin homolog
qRT-PCR	Quantitative real time polymerase chain reaction
RASTER	Microarray prognostics in breast cancer
RB1	Retinoblastoma tumour suppressor protein
RECQL4	RecQ Like helicase 4

RFS	Recurrence free survival
RNA	Ribonucleic acid
ROR	Risk of recurrence
RP	Rank products
RR	Relative risk ratio
RTKs	Receptor tyrosine kinases
S-phase	Synthesis phase
SD	Standard deviation
SERDs	Selective oEstrogen Receptor Down-regulators
SERM	Selective oEstrogen Receptor Modulator
SET	Sensitivity to Endocrine Therapy
SNAI1	Zinc finger protein
SoFEA	Study of Faslodex with or without concomitant Armidex vs Exemestane following progression on non-steroidal Aromatase inhibitors
SOFT	Suppression of Ovarian Function Trial
SPDEF	SAM Pointed Domain Containing ETS transcription factor
SPON1	Spondin 1
SPP1	Secreted phosphoprotein 1 or osteopontin
TAILORx	Trial Assigning Individualised Options for Treatment
TCGA	The Cancer Genome Atlas Network
TDLU	Terminal duct lobular unit
TEXT	Tamoxifen and Exemestane Trial
TF	Transferrin
TFF	Trefoil factor
TNBC	Triple negative breast cancers
TNFSF10	Tumour necrosis factor superfamily member 10
TNM	Tumour Node Metastases
TOP2A	Topoisomerase 2 alpha
TP53	Tumour protein P53
TTF	Transcription termination factor
UK	United Kingdom
USA	United States of America
VEGF	Vascular endothelial growth factor
VWF	Von Willebrand Factor
WT	Wild type
XBP1	X-box binding protein 1
ZEB1	Zinc finger Ebox binding

1 Background

1.1 Breast cancer - incidence and survival

Breast cancer is the most common female cancer in Scotland, with women currently facing a lifetime risk of 1 in 8.4 of developing the disease. In 2014, 4,610 new cases were diagnosed, representing 28.3% of all female cancers and 14.5% of male and female cancers [1]. Overall incidence is increasing, in part reflecting an extension of the Scottish Breast Screening Programme upper age limit to 70, as of April 2003, and also an increased uptake of breast screening services [2]. Despite increasing incidence, overall mortality is decreasing, representing the improved treatment options that are available and the detection of tumours at an earlier stage of disease. In 2014 breast cancer in Scotland contributed to 976 deaths, representing 12.6% of mortality from all female cancers, figure 1-1.

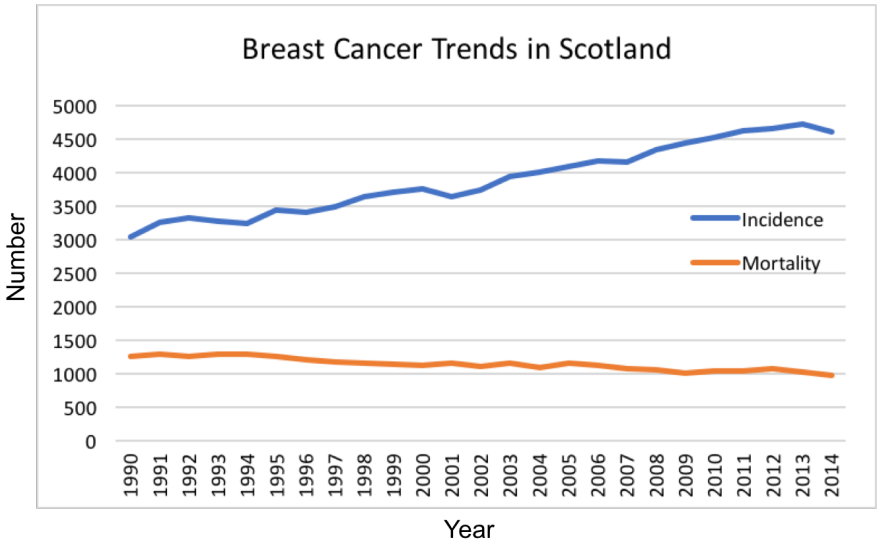

Figure 1-1. Annual incidence and mortality of breast cancer in Scotland Constructed from data available at Information Services Division Cancer Statistics, www.isdscotland.org/Health-Topics/Cancer/Cancer-Statistics/Breast/#cancer-of-the-breast

1.2 Breast anatomy

The breast is composed of a series of ducts extending peripherally and branching multiply to secretory glands (acini). 15-20 segments or lobules containing acini drain to around 12 main ducts at the nipple. Ducts branch repeatedly until giving rise to the terminal duct lobular unit (TDLU), figure 1-2A. It is here carcinomas arise. The glandular component of normal breast

fluctuates with hormonal change throughout the menstrual cycle, pregnancy and the menopause. The remaining breast tissue is composed of fatty and fibrous stroma. After the menopause the relative proportion of glandular tissue is less and stroma more [3]. The ducts and glands are lined by 2 layers of epithelium supported by a basement membrane, figure 1-2B.

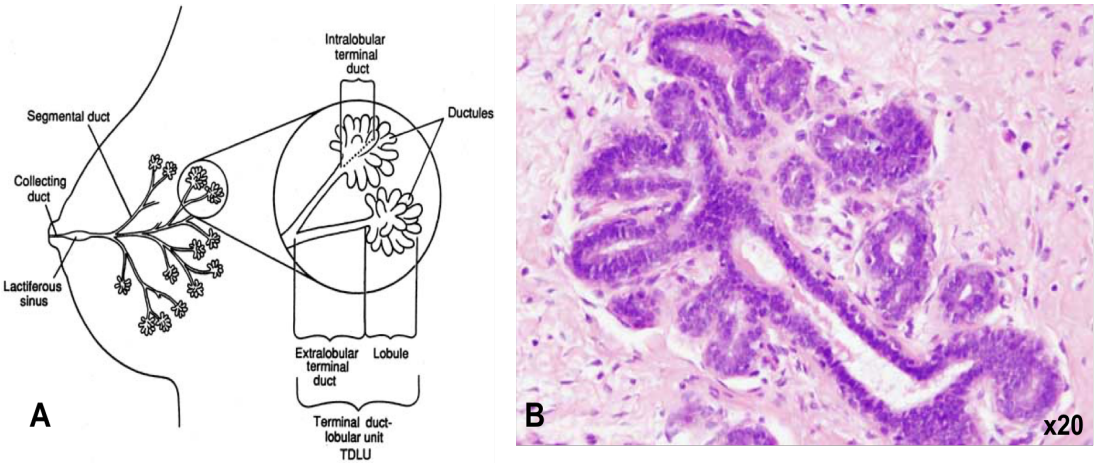

Figure 1-2. Breast lobe and terminal duct lobular unit

A: Schematic representation of breast ducts and lobules, from Hindle WH Breast Care p34, 1999.

B: Haematoxylin and eosin stained FFPE section of normal breast microscopy demonstrating TDLU, image courtesy of Dr Jeremy Thomas, Lead Consultant Pathologist, Edinburgh Breast Unit.

1.3 Pathology of breast cancer

Breast cancer is a spectrum of diseases with varying clinicopathological features and outcomes. In assessing patients with breast cancer, we must identify features of prognosis, risk of recurrence, and markers predictive of response, in order to guide clinical decisions on treatment.

1.3.1 Carcinoma in Situ

Carcinoma in situ is a pre malignant change in the breast where neoplastic proliferation of cells occurs in the epithelium of the TDLU but does not breach the basement membrane [4]. It is further classified as ductal (DCIS) or lobular (LCIS). LCIS exists within a spectrum of lobular neoplasia (LN), which also encompasses atypical lobular hyperplasia (ALH). Distinguishing LCIS from ALH can be challenging. LN is associated with development of

DCIS or invasive breast cancer in the order of around 8-9% in LCIS and 2-3% in ALH [5]. Classic low and intermediate grade LCIS and pleomorphic LCIS should be treated by excision, however there may be a role for observation only in isolated ALH.

DCIS often coexists with invasive disease and left untreated has potential to become invasive breast cancer [6]. Grading is based on cytonuclear staining and degree of necrosis into low, intermediate and high grade which corresponds to prognosis [7]. It is frequently associated with periductal and luminal microcalcifications seen on mammography, often in the screening programme, where it may occur in isolation.

1.3.2 Invasive breast cancer

70-75% of all breast cancers are invasive ductal carcinoma (IDC) of no special type. Invasive lobular carcinoma (ILC) accounts for a further 10-15% and the remainder are smaller histopathological subtypes including tubular, cribriform, mucinous, medullary-like, apocrine, metaplastic or mixed. Ductal and lobular breast cancers behave with distinct clinicopathological features. Histologically ILC is recognised by small round monomorphic cells in single file with pagetoid spread. There is often no significant surrounding invasion or connective tissue response [8]. This diffuse growth means there may be no distinct mass, making diagnosis clinically [9] and mammographically difficult [10]. ILC is more likely to be multifocal, multicentric [11] and bilateral [12]. LN and ILC are characterised by the loss of the intercellular adhesion molecule E-Cadherin expression [13], figure 1-3.

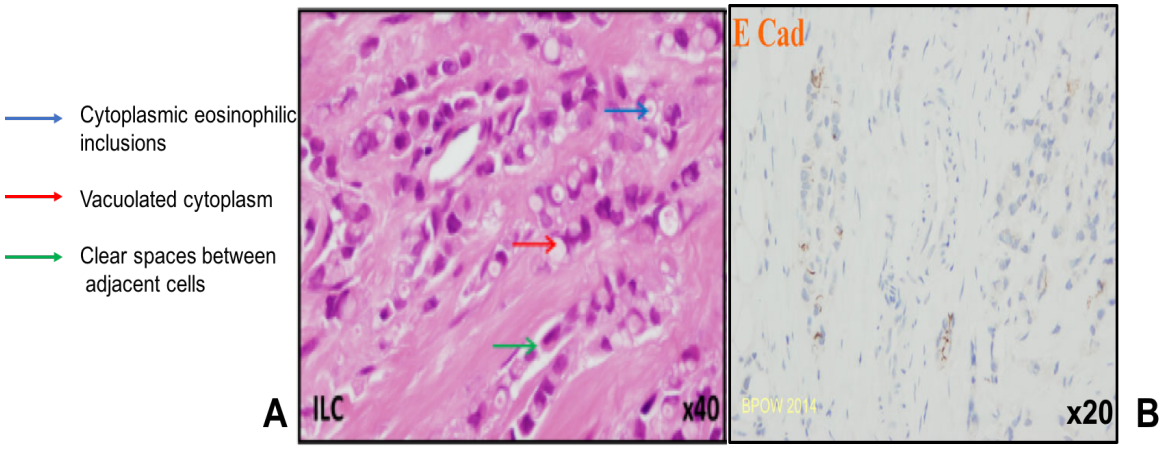

Figure 1-3. Invasive lobular carcinoma

A: H&E of classical ILC with distinguishing features highlighted.

B: Lack of immunohistochemical staining of E-Cadherin. Images - Dr Jeremy Thomas

1.3.3 Grading breast cancer

Elston and Ellis proposed the current internationally accepted grading system where reproducible quantitative evaluation of three morphological features; namely acinar formation, nuclear atypia or pleomorphism, and mitotic count are assessed and used to calculate an overall grade of 1, 2 or 3 for each tumour, providing important prognostic information [14].

1.3.4 Lymphovascular invasion

Lymphatic or blood vessel invasion, or both, is an indicator of poorer recurrence free and overall survival, independent of lymph node involvement or other tumour features [15].

1.3.5 Nodal status

Lymph node involvement is one of the most important prognostic factors in breast cancer, with prognosis deteriorating with increasing number of positive nodes [16]. Sentinel lymph node biopsy is the accepted method of staging the clinically and radiologically node negative axilla. Axillary lymph node dissection, with its associated morbidities, can be safely avoided in a negative sentinel node, and in patients with early breast cancer and a positive sentinel node [17]. Macrometastases are greater than 2mm diameter, micrometastases are 0.2-2mm diameter, and clusters of tumour cells measuring less than 0.2mm are termed isolated tumour cells. Micrometastases are associated with poorer prognosis [18], but the impact of isolated tumour cells detected in sentinel node biopsy is comparable to node negative disease [19] and therefore current Tumour Node Metastases (TNM) staging classify this as such. Extra nodal extension and invasion of adjacent lymphatics are also associated with poorer outcome [20].

1.3.6 Predictive Immunohistochemistry

75% of invasive breast cancers express oestrogen receptor alpha (ER+), a favourable prognostic factor and a strong predictor of response to endocrine therapies. Levels of ER and the progesterone receptor (PR) are assessed by the Allred Score, based on proportion and intensity of immunohistochemistry (IHC) staining in the nucleus, as 0 to 8, with scores of 2 or above being considered positive. Expression of ER and PR not only predict response to endocrine therapies but also have a prognostic significance. Moderate to poor ER expressing tumours (Allred <6) have a poorer prognosis than those which are ER-rich (Allred 7-8) [21]. Similarly, higher expression of PR is associated with more favourable outcome also [22]. In ER+ / PR- disease around 40-60% of patients derive benefit from endocrine therapy, however in ER+ / PR+ disease around 75% will benefit [23]. In ER- / PR+ disease around 40% will benefit [24]. Human epidermal growth factor 2 (HER2), an oncogene protein, is positive in

15-20% of invasive cancers and is associated with poorer outcome [25] although its presence is predictive of response to numerous immunotherapies including trastuzumab (a monoclonal antibody against HER2), lapatinib (tyrosine kinase inhibitor), pertuzumab (HER2 / HER3 dimerisation inhibitor) and trastuzumab emtansine (T-DM1), an antibody-drug conjugate which binds to HER2 and releases cytotoxic emtansine into the cell [26]. HER2, a transmembrane tyrosine kinase receptor, is assessed by IHC as 0, 1+ (negative), 2+ or 3+ (positive). Samples of 2+ are further assessed by fluorescence in situ hybridisation (FISH) to determine gene copy numbers to classify as positive or negative.

1.3.6.1 Ki67

Ki67 is a nuclear antigen and marker of proliferation which can be assessed by IHC. It is a valid prognostic indicator [27] and a marker of response to neo-adjuvant (pre-operative) chemotherapy [28] and endocrine therapy [29], when measured after an initial period of treatment, which can be as short as two weeks [30]. Ki67 has also been demonstrated as a marker of poorer recurrence free survival in subgroup analyses of node-negative, node-positive and untreated patients in a meta-analysis of 12,155 patients from 46 studies [31]. There has been difficulty establishing a reproducible and reliable method of measuring Ki67. There is risk of pathologist and institution dependent bias, and there is wide discrepancy about absolute values to be considered as 'high' or 'positive' as opposed to 'low' or 'negative'. The International Ki67 in Breast Cancer Working Group have developed recommendations for the gold standard method of assessing Ki67 in research trials [32]. This is favourable over semi quantitative imaging techniques, such as automated quantitative analysis (AQUA) [33], Automated Cellular Imaging System (ACIS) [34], or Scanscope [35]; which are validated methods of measurement of Ki67 expression, but are not routinely available in all centres.

1.4 Microarray technology and molecular pathology of breast cancer

Advances in high-throughput genomic technologies have augmented and reformed our understanding of breast cancer [36, 37]. Breast cancer is a highly complex and heterogeneous disease, highlighted by the numerous differences in clinicopathological features and varying responses to therapy seen in different tumours and patients. The high-throughput technologies provide simultaneous measurement of thousands of DNA sequences, RNA transcripts, peptides and metabolites giving a holistic overall portrait of complex tumour biology. Gene expression profiling in particular has been applied to many areas of research, with the potential to identify new targets for treatment, mechanisms of resistance and to improve on current tools for the analysis of prognosis [38, 39]. Broadly, a 'top-down' or 'bottom-up' approach to gene

expression analyses may be employed. Initial classification used a top-down approach, whereby samples of tumours from many patients were profiled, and candidate gene signatures which clustered together were identified, which could discriminate between patient groups based on similarities across thousands of genes or active pathways. In bottom-up analyses, a single or multigene predictor derived from *in vivo* or *in vitro* experiments corresponding to a phenotype, or resistance to a drug, is then applied to breast cancer samples [40].

1.4.1 Gene expression profiling intrinsic subtypes

Perou *et al* hypothesised that the phenotypic variation in breast cancers may be mirrored with diversity in gene expression, and using unsupervised hierarchical clustering on cDNA microarrays from 42 patients, proposed a molecular taxonomy of four molecular subtypes of breast cancer, based on differential expression of 496 intrinsic genes [41]. The four subtypes; basal-like, normal-like, *ERBB2*-enriched and luminal, were further classified into five subtypes with the distinction of a luminal A and poorer prognostic luminal B subtype by Sorlie *et al* [42] in 2001. They recognised each subtype was prognostic and later demonstrated subtype classification was reproducible across multiple microarray platforms [43]. Each subtype has distinct clinical outcomes and pathological features, figure 1-4.

1.4.1.1 Luminal A

Luminal A is the most commonly occurring subtype, accounting for 54% of all breast cancers [44]. It has high expression of *ESR1*, the gene encoding ERα, and a molecular profile resembling the luminal epithelial component of breast tissue; including expression of luminal cytokeratins 8 and 18, and genes associated with ER activation, including GATA binding protein 3 (*GATA3*), X-box binding protein 1 (*XBP1*), trefoil factor 3 (*TFF3*), hepatocyte nuclear factor 3 (*HNF3*), oestrogen-regulated *LIV-1*, and cyclin D1 (*CCND1*). Tumours are of lower grade and have a favourable prognosis [45] with a 10 year recurrence free survival of over 80% [46].

1.4.1.2 Luminal B

Luminal B tumours have lower expression of ER related genes and higher expression of proliferation associated and cell cycle genes including RecQ Like helicase 4 (*RECQL4*), cyclin B1 (*CCNB1*), MYB proto-oncogene like 2 (*MYBL2*), ASH2 like (*ASH2L*), B related factor 2 (*BRF2*), phospholipase *DDHD2*, fibroblast growth factor receptor 1 (*FGFR1*), leucine zipper and EF hand containing protein (*LETM2*), *LSM1*, and phosphatidate phosphatase (*PPAPDC1B*) [47]. Tumours are ER+, high grade with high Ki67. Gene set enrichment

analysis (GSEA) has identified activation of growth factor signalling, including *ERBB2*, however only 10-20% of tumours are positive for HER2 by IHC [48]. They respond less well to endocrine therapy than luminal A tumours, and less well to chemotherapy than basal and HER2 subtypes [49]. Overall they have a poorer prognosis than luminal A with increased risk of early relapse [50] and poorer disease free survival. Both Luminal A and B have a predisposition to metastasise to bone and pleura [51].

1.4.1.3 HER2-enriched

HER2 tumours are characterized by high expression of several genes in the *ERBB2* amplicon at 17q22.24 including *ERBB2*, Growth factor receptor bound proteins (*GRB7*), and post GP1 attachment to proteins (*PGAP3*) [41, 42]. They are more likely to be grade 3 and 40-80% harbour a mutation in the tumour suppressor gene *TP53* [52]. HER2 subtype has a poorer prognosis than luminal disease, but is sensitive to anthracycline and taxane based neoadjuvant chemotherapy with higher levels of pathological complete response (pCR) than in luminal tumours [53]. It is important to realise that not all tumours deemed HER2+ by IHC and FISH are HER2 enriched at transcription level, and a proportion of these are luminal subtype [54].

1.4.1.4 Basal-like

Basal-like tumours are ER- PR- HER2- (triple negative breast cancers, TNBC), and are high grade, highly proliferative with a poor prognosis [42]. They account for 10-25% of all breast cancers. Expression profiles resemble basal epithelial cells and normal breast myoepithelial cells with high expression of cytokeratins 5, 6, 14 and 17 [41], metallothionein, integrin B4, P-cadherin, laminin, and fatty acid binding protein 7 [46, 55]. Incidence is higher in African American women, especially those premenopausal [56]. Typically tumours present as a large rapidly growing mass and radiologically can be ill defined, lobulated, mixed cystic and solid [57]. The majority of tumours in *BRCA1* mutation carriers are of basal phenotype [58] and up to 80% of basal tumours have a *TP53* mutation [59]. EGFR is over expressed in 40% [60] which may have a role in resistance to chemotherapy.

1.4.1.5 Normal-like

The normal-like subtype expresses genes seen in adipose tissue, fibroadenomata and normal breast tissue including fatty acid binding protein and PPAR gamma [41]. They can be ER+ or ER-, and may express some basal epithelial genes therefore are difficult to distinguish from basal-like tumours by IHC only [61], although some researchers have adopted ER- PR- HER2-

CK5/6- EGFR- as being diagnostic of normal-like. It does however have a much more favourable outcome than basal-like breast cancer [62].

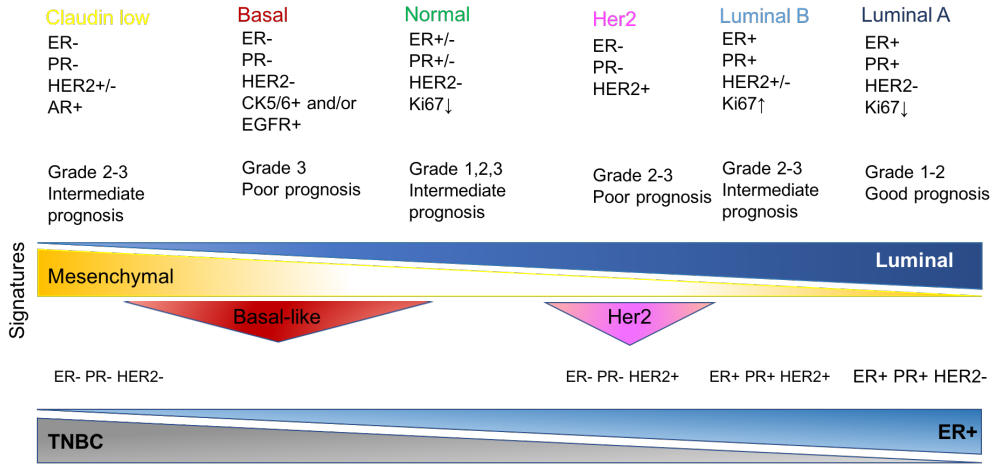

Figure 1-4. Molecular subtypes of breast cancer

Spectrum of breast cancer and associated clinical features. It should be noted that classifying tumours based on their IHC hormone receptor status does not always correspond to the appropriate molecular subtype, as defined by mRNA microarray, and may be an oversimplification; eg a proportion of tumours HER2+ by IHC will be luminal subtype.

AR – androgen receptor, CK – cytokeratin

The Sorlie intrinsic subtypes have been widely adopted as a gold standard taxonomy in the breast cancer field, although alternative subtypes have also been proposed.

Sotiriou *et al* identified 6 subtypes based on expression of 706 cDNA probes in 99 breast cancer specimens, including a third luminal subtype, 1 HER2-enriched, and 2 basal-like groups.

Luminal 1 tumours had higher expression of *c-kit*, hepatocyte growth factor (*HGF*), insulin-like growth factor binding-protein 3 (*IGFBP-3*), activating transcription factor 3 (*ATF-3*) and components of activating protein (*AP-1*) transcription factor, and lower expression of cell growth related genes, including topoisomerase 2α (*TOP2A*), mitotic kinesin-like protein (*MKLP)-1*, proliferating cell nuclear antigen (*PCNA*), cell division control protein 2 homolog (*CDC2*), *BUB1* and *MAD2L1*; and were associated with 80% 10-year recurrence free survival (RFS).

Luminal 2 subtype had higher expression of protein tyrosine phosphatase type IVA (*PTP4A1*), tumour necrosis factor receptor associated factor 3 (*TRAF3*), *RAD21* and *BRCA1*-associated protein, and lower expression of fibroblast growth factor receptor (*FGFR1*), chemokine receptor (*CXCR4*), *ATF-3* and vascular cell adhesion molecule 1 (*VCAM-1*); with a 10-year RFS of 40%.
Luminal 3 subtype had intermediate 10-year RFS of 60%.
Basal 1 subgroup had higher expression of matrix metalloproteinase-7 (*MMP7*) and cell growth-related and proliferation genes, including *TOP2A*, mitotic feedback control protein 2 homolog (*MADP2*), *CDC2* and *PCNA*.
Basal 2 tumours had higher expression of transcription factor AP-1 components, including c-Fos, c-Jun and Fos-B, and overexpression of transforming growth factor beta receptor 2, *ATF-3*, caveolin 1 and 2, and *HGF* [46].

A further subtype of TNBC; the claudin-low group, characterised by low expression of claudin genes, notably 3, 4 and 7, and also E-cadherin [63], all of which are involved in cell-cell junctions, has been identified. Claudin-low tumours are similar but distinct from basal-like tumours. They have immune infiltrates, features of stem cells and epithelial mesenchymal transition, including zinc finger Ebox binding (*ZEB1*) and zinc finger protein (*SNAI1*) [63].

Lehmann *et al* proposed 6 subtypes of TNBC; 2 basal-like, 1 immunomodulatory, 1 mesenchymal, 1 mesenchymal stem-like and 1 luminal androgen receptor.
Basal-like 1 subtype are enriched in cell cycle and cell division components and pathways with high proliferation.
Basal-like 2 subtype are enriched for pathways in growth factor signalling, glycolysis and gluconeogenesis, and growth factor receptors.
Immunomodulatory subtype is enriched in immune processes, including immune cell and cytokine signalling, antigen processing and presentation and complement cascade.
Mesenchymal subtype has high expression of cell motility components and pathways, ECM receptor interaction and cell-differentiation pathways.
Mesenchymal stem-like subtype is similar to mesenchymal, but also has genes relating to growth factor signalling pathway components and processes. It has low expression of claudins 3, 4 and 7 (as in claudin-low subtype).
The luminal androgen receptor subtype is ER- but has luminal gene expression patterns [64].

The molecular apocrine breast cancer subgroup is ER- and also characterised by androgen receptor (AR) expression and activation of AR signalling, including protein coding genes *FOXA1*, *AGR2*, and activated leukocyte cell adhesion molecule (*ALCAM*), transcription factor *SPDEF*, and *TTF3* [65].

1.4.2 Genomic expression profiling subtypes

Curtis *et al* described 10 integrative cluster (IntClust, IC) breast cancer subtypes in the Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) study; based on integrated genomic copy number and transcriptomic profiling in 2,000 patients. Clusters correlated with distinct clinical outcomes. Inherited genetic variants and acquired somatic copy number aberrations (CNAs) were associated with expression in >39% of genes; with most influence derived through CNAs acting in *cis* (influencing its own expression) or *trans* (affecting other gene expression also). They highlighted several genes as putative breast cancer tumour suppressor genes, including *PPP2R2A*, *MTAP* and *MAP2K4*. They proposed further sub-classification within the luminal subtypes.

ICs-3,7 and 8 are predominantly luminal A, with IC-3 lacking copy number and genomic variants, IC-7 having 16p gain / 16q loss and 8q amplification, and IC-8 having 1q gain / 16q loss.

IC-2 is a high risk luminal subtype, which harbours 11q13 / 14 aberrations, affecting *CCND1* and several oncogenes.

ICs-1, 6 and 9 were predominantly luminal B subtype; associated respectively with 17q23 / 20q *cis* aberrations; 8p12 *cis* aberrations and 8q *cis* aberrations / 20q amplifications.

IC-5 incorporates an expanded *ERBB-2* amplified group incorporating both ER+ and ER- tumours.

IC-10 are basal subtype and the most unstable genomically.

IC-4 included various transcriptional intrinsic subtypes but shared similar genomic features to IC-3 (lacking copy number and *cis*-acting alterations) [66].

The Cancer Genome Atlas Network (TCGA) integrated data from multiple platforms; genomic copy number arrays, DNA methylation, exome sequencing, mRNA, micro RNA, and reverse phase protein arrays to classify breast cancer into 4 subtypes; luminal A, luminal B, HER2-enriched and basal-like. Subtypes were associated with particular gene mutations; with luminal A harbouring 45% *PIK3CA* mutations, followed by other most frequent mutations in *GATA3*, *MAP3K1*, *TP53*, *CDH1*, *PIK3R1* and *MAP2K4*. Luminal B tumours had mutations in *PIK3CA* in 29% and *TP53* in 29%. Basal tumours had *TP53* mutations in 80%, but *PIK3CA*

mutations in only 9%. The HER2-enriched subtype was associated with *ERBB-2* amplification in 80%, mutations in *TP53* in 72% and *PIK3CA* in 39%, with lower levels of mutations of the luminal associated gene mutants, eg *PIK3R1* mutations in only 4% [67].

Molecular subtype classification in breast cancer is complex and far from standardised. Furthermore, whole genome arrays are costly and often require fresh frozen tissue samples; two factors which vastly limit their reproducibility in routine clinical care. To guide treatment, we must identify features of poor prognosis and those predictive of response to targeted therapies. Multiple classifiers have been proposed (see section 1.9), though not all are routinely used in UK clinical practice, and indeed not all are helpful in treatment decisions.

Broadly, three subtypes are used to guide treatment;
ER+ and / or PR+, treated with ER+ targeted endocrine therapy,
HER2+, treated with targeted anti-HER2 agents, and
TNBC, treated with chemotherapy.

1.5 Treatment of early breast cancer

Following diagnosis and staging of breast cancer, a treatment plan will be proposed. In early disease, this is likely to involve surgery, either as first treatment or increasingly, following a period of pre-operative or neoadjuvant therapy. Adjuvant (post-operative) treatments including chemotherapy, radiotherapy and anti-HER2 therapy, are offered to appropriate patients thereafter, figure 1-5. Neoadjuvant treatment can be used to downstage large or locally advanced breast tumours, increasing rates of breast conserving surgery thereby avoiding mastectomy [68]. Neoadjuvant chemotherapy has been established in this role since the 1970s and more recently targeted endocrine and anti-HER2 agents have also been used in appropriate patients [69, 70]. A further advantage is a unique *in vivo* observation of tumour response to treatment [71]. Taking sequential biopsies from the same tumour in this 'window of opportunity' can potentially identify molecular subtypes or markers associated with response and resistance. This may identify patients unlikely to respond to a certain drug, who require alternative or combination therapies, and ultimately spare them from unnecessary and ineffective treatment. Longer term prognostic information can also be derived in this very early period. Response in the neoadjuvant setting, particularly pathological complete response, is associated with a favourable prognosis with improved disease free and overall survival in the longer term; especially in HER2-positive and triple negative breast cancer subtypes [72].

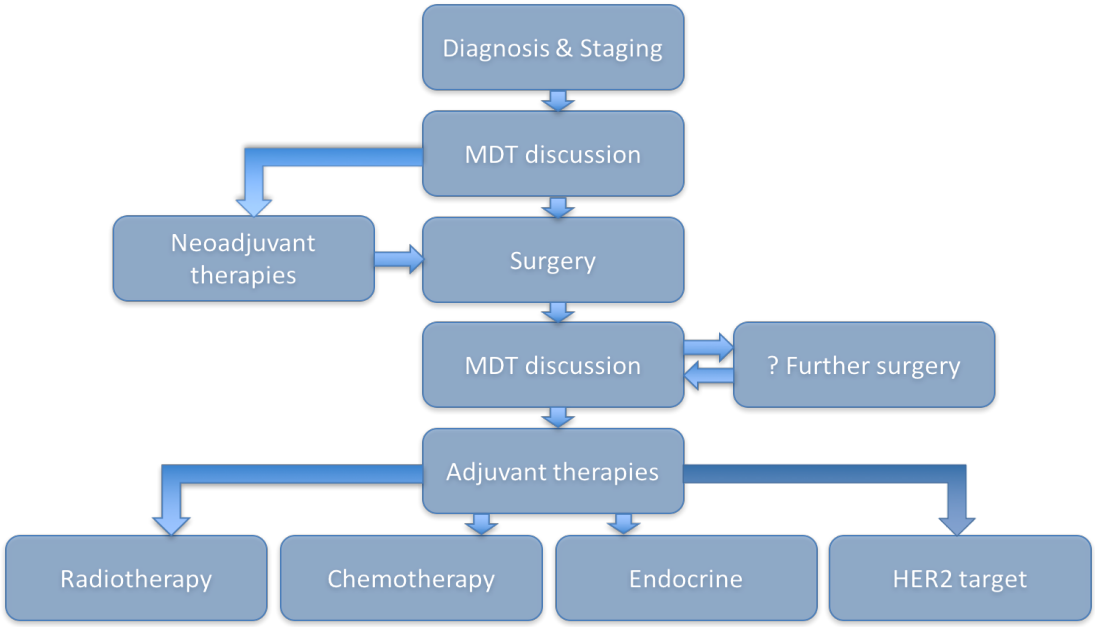

Figure 1-5. Treatment of early breast cancer
Treatment is decided on an individual basis reflecting patient host, tumour and stage of disease factors. Further surgery may be considered in women undergoing breast conservation surgery who have positive margins at the initial resection.
MDT – multi-disciplinary team.

The remainder of this discussion will focus on endocrine treatments and ER+ breast cancer. Most ER+ disease will respond to endocrine therapies. Our challenge as clinicians is to identify clinical and biological features of poor prognosis or prediction of resistance in order that we target these patients with additional treatment. Equally we must also identify patients with such good prognostic features they can be spared from toxic adjuvant chemotherapy which may offer little advantage.

1.6 Oestrogen and oestrogen receptor (ER) in breast cancer

The role of oestrogen in the pathogenesis of breast cancer was first recognised by George Beatson in 1896 [73]. His experimental oophorectomy was the first form of endocrine therapy. It was later recognised that oestrogen modulated its effects on target tissues through specific proteins, namely oestrogen receptors (ER) [74, 75]. There are two isoforms of ER, ERα and ERβ, with ERα being predominant in ER+ breast cancer pathogenesis and treatments [76].

The endogenous oestrogen, 17β-oestradiol (E2), enters cells and binds to the ligand binding domain (LBD) of ERα. There are two transcriptional activation domains of ERα, activating function (AF)1 in the amino terminal region, which is regulated by phosphorylation, and AF2 in the carboxy-terminal region, which is regulated by ligand binding [77]. AF1 and AF2 activate transcription independently and / or synergistically. E2 binds to the LBD of ERα (E2-ER) in a strong but reversible way and enters the nucleus. The LBD is encoded by around 300 amino acids and forms a wedge-shaped structure incorporating the ligand-binding pocket. When E2 binds at the LBD, ER activates in response undergoing conformational change, with dissociation of heat shock proteins and formation of a ligand-occupied ER dimer [78]. The conformational change in ER uncovers a surface that can recruit co-activators. It also results in dissociation of co-repressors [79]. The E2-ER binds as a dimer to small palindromic DNA sequences, known as oestrogen response elements (EREs) in the promotor regions of specific oestrogen regulated target genes (see figure 1-6). Co-activators bind histone acetyltransferases (HATs), leading to acetylation of histones and decondensation of chromatin, facilitating transcriptional activation. In its un-liganded state, ER is bound to corepressor complexes, which recruit histone deacetylases (HDACs) [80]. Their function is to maintain histones is a deacetylated state which will favour chromatin condensation.

ER can also interact with EREs through numerous transcription factors which help tether the receptor to DNA. The E2-ER is not directly bound to DNA, but interacts with other DNA bound proteins forming multiprotein complexes which augment binding of other transcription factors and prevent chromatin condensation. AP1 factors, such as JUN and FOS proteins, have been implicated in this role [81].

ER activation is also possible in the absence of a ligand, mediated by phosphorylation of ER due to stimulation by alternate growth factors. Phosphorylation at several sites can increase ER activity, including at serine 118 within AF1. Serine 118 is phosphorylated by the mitogen activated kinases (MAPK), ERK1 and ERK2, and also by CDK7 [82]. AKT, a downstream

target of PI3K, phosphorylates ER at serine 167 in AF1 [83], also resulting in ligand independent activation. PI3K can be activated by cross talk from numerous growth factor pathways, including EGFR, HER2 and insulin-like growth factor receptor (IGF1R), potentially causing E2 independent activation of ER.

Cyclin D1 is a direct transcription target of ER and other EGFR signalling pathways. Cyclin D1 activates cyclin dependent kinases (CDK) 4 and 6, and also activation of cyclin E and CDK2, both leading to downstream hyper-phosphorylation of retinoblastoma tumour suppressor protein (RB1) [84], thereby dissociating from and losing its inhibition of transcription factors, notably E2F family, activating genes required for progression from G1 to S phase of the cell cycle [85].

1.7 Endocrine therapy in ER+ breast cancer

Endocrine therapy in breast cancer targets either the oestrogen receptor, competitively binding or modulating ER to exert an antagonistic effect; or inhibits oestrogen biosynthesis to reduce circulating levels of E2 [86]. In premenopausal women, oestrogen is produced by the ovaries, whereas in postmenopausal women oestrogen is produced by enzymatic conversion of androgens (androstenedione and testosterone) to oestrogens (such as oestradiol and oestrone) by aromatase [87], figure 1-6. Aromatase is present most abundantly in adipose tissue, but also other sites including muscle [88], bone and brain. Aromatase is also present in normal breast and breast cancer tissue [89]. An alternative approach to endocrine therapy is required in pre and postmenopausal women.

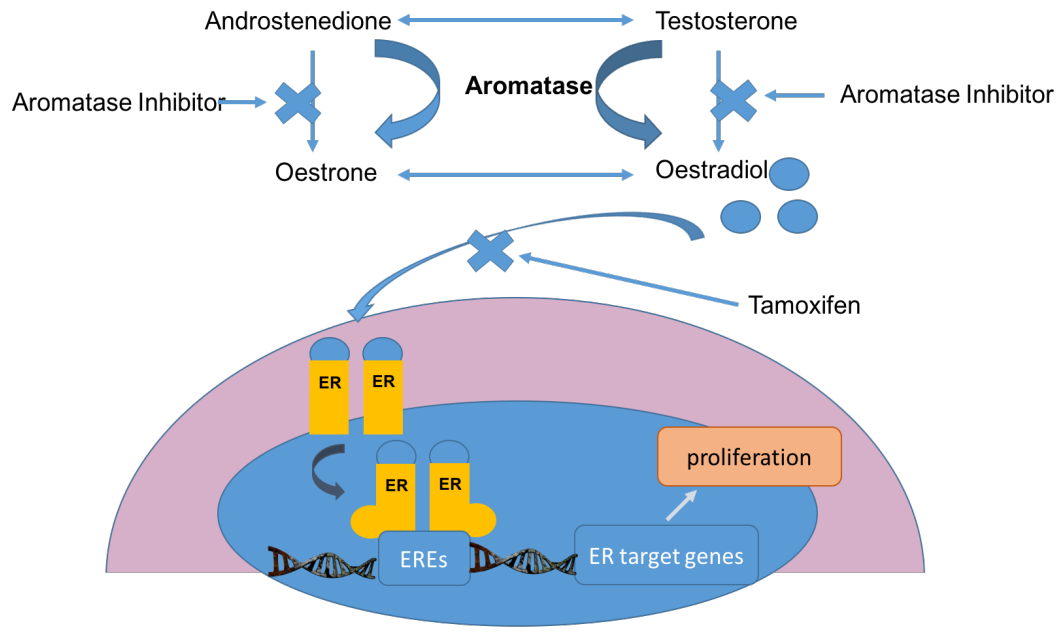

Figure 1-6. Oestrogen and ER in breast cancer, mechanism of action of endocrine therapies

Oestradiol binds to ER, receptor dimerises, undergoes conformational change and binds to oestrogen response elements (EREs) upstream of oestrogen responsive genes necessary for proliferation.

Aromatase inhibitors block the conversion of androgens to oestrogens. Tamoxifen competitively binds with ER.

1.7.1 Anti-oestrogens – SERMs

Tamoxifen is a Selective oEstrogen Receptor Modulator (SERM) which mimics oestrogen binding to the ligand binding domain of ERα and is the mainstay of endocrine therapy in ER+ breast cancer in pre and peri-menopausal women [86]. Binding of tamoxifen to the ER LBD activates a similar cascade as seen with E2, however the resulting conformational change in the receptor is different. Activation of AF1 does occur but not activation of AF2, resulting in partial agonist and partial antagonist activity. Tamoxifen bound ER interacts with co-repressors promoting their recruitment to AF2, and preventing recruitment of co-activators [90].

Tamoxifen became first established as a tumouristatic agent in advanced breast cancer [91] in the 1970s, where it was noted to prevent disease progression for 1-2 years. In the adjuvant setting, 5 years of tamoxifen reduces the risk of breast cancer death by one third, and reduces

breast cancer recurrence by one half at 15 years [92, 93]. 5 years of therapy is superior to 1-2 years [23]. Treatment is most effective in patients with high expression of ER [94]. The Adjuvant Tamoxifen: Longer Against Shorter (ATLAS) trial, demonstrated reduction in recurrence and improved survival at 15 years when tamoxifen was continued to 10 years compared with 5 [95], particularly during years 10-14. Toxicities, most commonly menopausal-like symptoms [96], can be problematic which can reduce compliance however. Tamoxifen is also associated with a relative risk ratio (RR) of twofold for development of venous thromboembolism (higher RR of 3.5 in first 2 years of treatment) [97], and also increased risk of endometrial hyperplasia and endometrial carcinoma of about 2.5 times [87], due to the presence of both agonist and antagonist properties specific to different tissues [98].

Raloxifene, toremifene and arzoxifene are alternative SERMs. Raloxifene was initially licensed for osteoporosis prevention and treatment in postmenopausal women, but was also found to reduce the risk of developing breast cancer in the Multiple Outcomes Raloxifene Evaluation (MORE) study by 76% (13 of 5,129 patients with raloxifene, 27 of 2,576 with placebo; RR 0.24; 95% CI 0.13-0.44, $p<0.001$) [99]. Raloxifene, similar to tamoxifen, causes a twofold relative risk of thromboembolism [100], however no increased risk of endometrial cancer, because unlike tamoxifen, raloxifene lacks the agonist activity of uterine ER [101].

There is limited evidence on the role of neoadjuvant endocrine therapy in premenopausal women. A study assessing 7 days of pre-operative tamoxifen in 44 patients (58% post-menopausal, 32% pre or peri-menopausal) found a mean decrease in Ki67 of 40% (95% CI 29-63%) [102]. The STAGE trial compared neoadjuvant anastrozole with tamoxifen in premenopausal women receiving goserelin (Gonadotrophin Releasing Hormone Agonist, GnRH) and found more patients had complete or partial response with anastrozole than tamoxifen (70.4% vs 50.5%; difference 19.9%, 95% CI 6.5-33.3, $p=0.004$) [103]. Many premenopausal patients will have features of adverse prognosis and be considered for neoadjuvant chemotherapy rather than endocrine therapy (see section 1.9).

1.7.2 Anti-oestrogens - SERDs

Unlike SERMs the role of SERDs has been established in postmenopausal rather than premenopausal women. Fulvestrant, (Faslodex), is a Selective oEstrogen Receptor Downregulator (SERD) which binds to ER and dissociates receptor proteins, in doing so accelerating receptor degradation so the rate of dimerization and nuclear localisation of the fulvestrant-ER complex is reduced. Binding to ERE is reduced, thereby transcription of ER

regulated genes are reduced [104]. It has poor oral bioavailability and is therefore administered by monthly intramuscular injection, following initial loading doses. In advanced disease, in patients previously treated with tamoxifen, fulvestrant is at least as effective as the aromatase inhibitor, anastrozole (see section 1.6.3) in delaying time to disease progression and treatment failure [105], at a monthly dose of 250mg. Evidence of a dose related response was apparent with a higher dose of 500mg achieving near 100% ER down-regulation compared to 70% down-regulation with a 250mg dose [106]. Indeed, increasing the dose to 500mg compared to 250mg improved survival by a median of 4.1 months in the Comparison of Faslodex in Recurrent or Metastatic Breast Cancer (CONFIRM) trial of 736 women, who tolerated the higher dose well with no increased reported adverse events [107]. The Fulvestrant First-Line Study Comparing Endocrine Treatments (FIRST) trial found higher dose 500mg fulvestrant was non-inferior to anastrozole in time to disease progression in advanced and metastatic disease, and found higher dose fulvestrant was well tolerated, with 11% adverse events but only 3% discontinuing treatment [108]. At this planned initial end point the median time to progression had not been reached for fulvestrant and a subsequent follow up analysis confirmed significantly improved time to progression with fulvestrant over anastrozole (23.4 vs 13.1 months, p=0.01) [109]. Recently, the fulvestrant 500mg versus anastrozole 1mg for hormone receptor-positive advanced breast cancer (FALCON) trial, confirmed fulvestrant to have superior efficacy in advanced and metastatic endocrine naive patients compared to anastrozole (median progression free survival 16.6 vs 13.8 months, p=0.048) [110]. This double-blind randomised multicentre trial of over 500 patients demonstrated slightly higher adverse events in the fulvestrant group (arthralgia 17% vs 10%) however similar discontinuation rates (7% vs 5%). Interestingly there was no improvement in overall survival. Most patients with metastatic ER+ disease will however have had one or more prior endocrine therapy, so further evaluation of fulvestrant in these patients will be necessary.

1.7.3 Ovarian suppression

Ovarian suppression (OS) in premenopausal women can be achieved medically by gonadotrophin releasing hormone agonists (GnRH); surgically by oophorectomy, or by ablation with pelvic irradiation. Patients who experience chemotherapy-induced amenorrhoea in the treatment of ER+ breast cancer have improved survival [111]. The Suppression of Ovarian Function Trial (SOFT) did not however show improved disease free survival with the addition of OS to tamoxifen compared to tamoxifen alone across all unselected premenopausal ER+ patients [112]. In a cohort treated with exemestane plus OS however there was 7.7% improvement in recurrence free survival at 5 years compared to tamoxifen alone. Similarly,

the Tamoxifen and Exemestane Trial (TEXT) found exemestane plus OS superior to tamoxifen plus OS by 4 percentage points in disease free survival at 5 years [113]. A striking subgroup analysis in SOFT revealed young women under age 35 at diagnosis had the most benefit from OS with a 5 year breast cancer free incidence of 67.7% in tamoxifen alone, 78.9% for tamoxifen plus OS and 83.4% for exemestane plus OS; strongly supporting the roll of OS in this high risk population [111]. The American Society for Clinical Oncology (ASCO) updated its guidance to recommend OS in all premenopausal women who would be considered for adjuvant chemotherapy because of high risk features [114].

1.7.4 Aromatase Inhibitors

Aromatase, a product of the *CYP19* gene, is a cytochrome P450 enzyme. The third-generation aromatase inhibitors are those currently utilised in clinical practice today, and include anastrozole (Arimidex), letrozole (Femara) and exemestane (Aromasin), all administered in a once daily oral preparation. Anastrozole and letrozole are non-steroidal and bind reversibly to the heme group of the aromatase enzyme at their triazole group. Exemestane is an aromatase inactivator, a steroidal androgen substrate analogue that binds competitively and irreversibly to aromatase [115]. All 3 drugs can supress whole body aromatisation *in vivo* in the order of 97-99%, corresponding to reduced levels of circulating oestrone, oestradiol and oestrone sulphate of 81-95%, 85-92% and 93-98% respectively [116, 117].

1.7.4.1 Aromatase Inhibitors in metastatic disease

Aromatase inhibitors (AIs) are superior to tamoxifen as first line therapy for postmenopausal women with ER+ breast cancer. This was first established in the metastatic setting via three key multicentre double blinded randomised controlled trials. Letrozole was significantly superior to tamoxifen in delaying time to progression in 907 patients (41 vs 26 weeks), time to treatment failure (40 vs 25 weeks) and objective response rate (30% vs 20%) respectively [118]. A further study found anastrozole superior to tamoxifen in time to progression (11.1 vs 5.6 months) with clinical benefit seen in 59% of patients with anastrozole compared to 46% of those with tamoxifen in 353 patients. They also demonstrated fewer adverse events with anastrozole compared to tamoxifen (4.1% vs 8.2% thromboembolism; 1.2% vs 3.8% vaginal bleeding) [119]. A further trial comparing anastrozole with tamoxifen in 668 women, found the drugs were comparable, but anastrozole not superior to tamoxifen, with median time to progression of 8.2 months with anastrozole vs 8.3 months with tamoxifen. They also found both drugs comparable in objective response (32.9% of patients with anastrozole vs 32.6% of those with tamoxifen). Similarly they demonstrated lower adverse events with anastrozole

compared to tamoxifen (4.8% vs 7.3% thromboembolism, and 1.2% vs 2.4% vaginal bleeding respectively) [120] and therefore recommended use of anastrozole over tamoxifen. The reason this study failed to confirm superiority of aromatase inhibitors over tamoxifen as the others did is unclear. There was a higher proportion of patients with unknown ER status included however, and retrospective subgroup analysis of those known to be ER+ showed trends in favour of anastrozole over tamoxifen.

1.7.4.2 Aromatase Inhibitors in adjuvant setting

In the adjuvant setting, the Arimidex, Tamoxifen, Alone or in Combination (ATAC) trial established anastrozole as superior to tamoxifen in 9,366 women with early breast cancer. Disease free survival was significantly prolonged at 68 months (575 events with anastrozole, 651 with tamoxifen, hazard ratio 0.87, 95% CI 0.78-0.97, p=0.01), as was time to recurrence (402 events vs 498 events, HR 0.79, 95% CI 0.70-0.90, p=0.0005) [121, 122]. Distant metastases and contralateral breast cancers were also significantly reduced. The combination arm was discontinued due to non-superiority over tamoxifen alone.

Similarly the Breast International Group (BIG) 1-98 trial confirmed superiority of 5 years of adjuvant letrozole over tamoxifen with an 18% reduction in risk of an event (HR 0.82; 95% CI 0.71-0.95, p=0.007) [123, 124].

MA-17 was a double-blind randomised controlled trial of 1,918 women who had completed 5 years of an adjuvant aromatase inhibitor, comparing 5 further years of letrozole vs placebo. Disease free survival was superior in patients receiving 10 years of AI, over 5 years AI plus 5 years placebo (HR 0.66, 95% CI 0.48-0.91, p=0.01) [125]. There was no difference in overall survival however and it is worth noting 80% of these patients had also had prior tamoxifen before starting AI; most of them for duration of 4.5-6 years.

A further approach is a sequence of adjuvant tamoxifen switching to an aromatase inhibitor after 2-3 years of treatment. The ideal sequence and duration is still debated. A large trial of 4,742 patients confirmed superiority, across all events, of 2 years tamoxifen switched to exemestane, over 5 years tamoxifen alone (HR 0.68, 95% CI 0.56-0.82, p<0.001), although no significant difference in overall survival was found [126]. Similarly, in 5,187 women treated with adjuvant tamoxifen for 5 years, a switch to letrozole at that point improved disease free survival over placebo (93% vs 87%, p<0.001) [127]. A switch to anastrozole after completion of two years of tamoxifen is also associated with improved progression free

survival with a 40% reduction in events over continuing tamoxifen for five years of endocrine therapy [128]. The adjuvant tamoxifen and exemestane in early breast cancer (TEAM) trial demonstrated superiority of 2-3 years tamoxifen switched to exemestane over tamoxifen alone, and comparable results of the switch approach to 5 years of monotherapy with exemestane, suggesting both switch and monotherapy approaches are reasonable treatment options [129].

The benefit of AIs over tamoxifen in postmenopausal ER+ women is well established. However, interestingly this has not translated into improved overall breast cancer specific survival [130, 131]. AIs generally are better tolerated than tamoxifen with fewer gynaecological side effects, however the incidence of fractures, arthralgia and osteoporosis are increased [132].

1.7.4.3 Aromatase Inhibitors in neo-adjuvant setting

A meta-analysis of four trials including 1,160 patients confirmed superiority of neoadjuvant AIs over tamoxifen in postmenopausal women, with increased clinical objective response rate (RR 1.29; 95% CI 1.14-1.47, $p<0.001$) and increased breast conserving surgery rate (RR 1.36; 95% CI 1.16-1.59, $p<0.001$) [133].

The P024 trial confirmed superiority of 4 months of neoadjuvant letrozole over tamoxifen with overall response rates of 55% and 36% respectively ($p<0.001$); and increased rate of breast conservation surgery (45% vs 35%, $p=0.022$) [134]. Letrozole also correlated with a greater reduction in mean Ki67 (87% in letrozole, 75% with tamoxifen) [135]. Letrozole has also been demonstrated as an effective primary endocrine therapy in elderly patients [136].

The Immediate Preoperative Anastrozole, Tamoxifen, or Combined with Tamoxifen (IMPACT) trial compared neoadjuvant tamoxifen, anastrozole or both for 3 months and found no significant difference in objective response rates between the groups but did demonstrate increased rate of breast conservation in the anastrozole group over tamoxifen group (46% vs 22%, $p=0.03$) [137]. In the Preoperative Anastrozole Compared with Tamoxifen (PROACT) trial, 3 months of neoadjuvant anastrozole and tamoxifen yielded similar response rates by caliper and ultrasound measures. This trial included patients undergoing neoadjuvant chemotherapy; and when excluding these a trend to improved response was seen with anastrozole (36.2% vs 26.5%, $p=0.09$) [138].

Similar to letrozole and anastrozole, exemestane is superior to tamoxifen in the neoadjuvant setting with improved clinical response rate (76% vs 40%, p=0.05) and increased breast conservation rate (36.8% vs 20%, p=0.05) [139].

The American College of Surgeons Oncology Group (ACOSOG) Z1031 compared the three third generation AIs for 16-18 weeks of neoadjuvant treatment and found no significant differences between anastrozole, letrozole and exemestane treated groups. Rates of breast conservation were improved with all AIs with no one drug being superior [140]. Similar rates of down regulation of ER, PR and reduced Ki67 have also been shown with short course (14 days) of anastrozole and letrozole with no significant difference between these drugs [141].

Most of the trials of neoadjuvant endocrine therapy have treated patients for a duration of 3-6 months. Prolonging neoadjuvant letrozole has also been shown to increase response rates with breast conservation increasing from 60% to 72% in women who continued treatment for longer than 3 months [142], supporting a longer treatment period.

1.7.4.3.1 Neoadjuvant endocrine therapy vs chemotherapy

Comparisons of neoadjuvant chemo and endocrine therapy are limited reflecting the fact that neoadjuvant chemotherapy is less effective in ER+ disease than ER- in achieving pathological complete response (8% in ER+ cancers compared to 24% of ER-, p<0.001) [143]. The Neoadjuvant Endocrine vs Chemotherapy Trial (NEOCENT) was designed to answer this question, however the trial closed early due to slow recruitment therefore no significant conclusions can be drawn [144].

1.8 Molecular changes in response to neoadjuvant aromatase inhibition

Miller *et al* investigated changes in gene expression profiles in response to 14 days of neoadjuvant letrozole in 58 patients, and found consistent changes in 143 genes. 91 down-regulated genes were involved in proliferation, cell cycle progression and mitosis (including *CDC2*, cyclins B1 and B2, *CKS2*), and oestrogen related (including *KIAA0101*, *TFF1*, *TFF3*, *IGFR1*). Up-regulated genes were involved in connective tissue extracellular matrix (including *COL1A1*, *COL1A2*, *MMP2*, *DCN*), immune response and inflammation (*ILIR1*, *COLEC12*, *TNFSF10*), and cell adhesion (*CD36*, *CDH111*, *ITGB2*, *SRP*, *SPON1*, *DPT*) [145].

Mackay *et al* conducted a similar study comparing the effect of 2 weeks preoperative anastrozole and letrozole in 34 patients, and found very similar changes, with down-regulation

of oestrogen related and proliferation genes, and up-regulation of extracellular matrix remodelling genes, including collagens and small leucine-rich proteoglycan family members. Similar changes were seen in response to both letrozole and anastrozole. They derived the Global Index of Dependence on oEstrogen (GIDE), from the number of genes with greater than two fold change between baseline and 14 days, which correlated strongly with change in Ki67 in response to treatment and negatively with *ERBB2* expression [146].

A further study by Miller, identified 205 co-variables, (69 at baseline, 45 at day 14, and 91 changed between baseline and day 14) which could differentiate between responsive and non-responsive tumours after 14 days of neoadjuvant letrozole [147]. In subsequent work, they were unable however to define consistent changes in gene expression in tumours non-responsive to neoadjuvant letrozole with some demonstrating similar down regulation of oestrogen related and proliferation associated genes as responsive tumours. This highlights the inherent heterogeneity of endocrine resistance in breast cancers [148].

Response to neoadjuvant endocrine therapy can be assessed clinically, with calipers, and surgeon opinion of achieving breast conservation; or radiologically with mammography, ultrasound or MRI measurements. As molecular changes are apparent after only 10-14 days of treatment [148] this presents an opportunity to identify features of response or resistance much earlier than would be apparent clinically [147, 149]. Sequential biopsy allows measures of molecular markers on treatment, including Ki67 [150], which can predict response to neoadjuvant endocrine therapy [151]. This equally correlates to long term outcome, as was first demonstrated in the IMPACT study, where 2 week on treatment Ki67 was predictive of recurrence free survival at median 37 months; Ki67>10%, 9 of 35 events (26%), Ki67<10% 13 of 118 events (11%), p=0.008 [137, 152]. This was also validated in the ATAC cohort [121, 122].

In estimating prognosis, numerous clinicopathological and biomarker studies have emerged. The opportunity to take sequential biopsies from the same tumour over time in response to treatment, has also heralded predictive biomarker studies (see section 1.9.3).

1.9 Prognostic and predictive factors

Clinical decision making in breast cancer requires robust and personalised stratification in order that patients are given appropriate drugs and spared from potentially toxic over-treatment that would offer little benefit [153]. In doing this we must identify markers of

prognosis, that is a measurable characteristic which provides likely outcome in a non-treated individual, to ensure those with higher risk disease are offered appropriate systemic therapies [154]. Predictive markers identify those who would likely benefit from a particular treatment, in terms of disease response or overall survival [155]; the most common in breast cancer being ER, expression of which is predictive of benefit from endocrine therapy. HER2 is both a marker of poorer prognosis and predictive of response to anti-HER2 targeted therapy. The classic clinicopathological prognostic and predictive features are discussed earlier in section 1.3. Further methods using clinicopathological features to guide clinical decisions are discussed below.

1.9.1 Clinicopathological prognostic estimates

1.9.1.1 Nottingham Prognostic Index

Nottingham Prognostic Index (NPI) is a numerical value calculated from tumour size, lymph node staging and tumour grade [156] into one of six prognostic groups; excellent, good, moderate I, moderate II, poor and very poor corresponding to increasing number [157]. NPI correlates well with long term outcome at 10-15 years [158], has been independently validated [159], and is easily reproducible with no added cost to standard measured pathology variables. The ability to identify high risk disease is comparable to gene expression profiling [160], however it does not take any account of tumour biology, therefore would not identify poor prognosis subgroups, such as small sized basal-like tumours [161] or HER2+ tumours. Attempts to combine IHC biomarkers with NPI have improved on this [162, 163] but undoubtedly could be further refined.

1.9.1.2 Adjuvant! Online

Adjuvant! Online (AO) predicts prognosis and likely net benefit of endocrine and chemotherapy [164], based on patient demographics and clinicopathological tumour features, calculated by actuarial analysis of multiple epidemiological and outcome data. AO has been demonstrated as effective as NPI for assigning prognostic group [165, 166], however is not reliable in the elderly, where it overestimates disease free and overall survival [167], or in *BRCA1* mutation carriers where it underestimates risk [168].

1.9.1.3 St Gallen Consensus Guidelines

In 2015 the St Gallen Consensus Conference expert panel agreed that molecular subtype can be determined on basis of IHC rather than RNA profiling or multigene classifiers [169] (see section 1.9.2) as follows:

luminal A: ER+ / PR+ / HER2- / Ki67 low,
luminal B: ER+ / PR+ / HER2- / Ki67 high or ER+ / PR+ / HER2+,
triple negative: ER- / PR- / HER2- and
HER2 enriched: ER- / PR- / HER2+.
The panel agreed that determination of subtype was necessary for treatment planning, however it was split on advising whether Ki67 or multi-gene assaying was always required in deciding to use chemotherapy in ER+ HER2- patients.

1.9.1.4 IHC4

IHC4 uses a formula incorporating IHC measures of ER, PR, HER2 and Ki67 to calculate an IHC4 score which correlates to prognosis [170]. IHC4 provides valuable additional prognostic information combined with standard clinicopathological measures. However in node negative, HER2 negative subgroups, it is inferior to gene assays, including PAM50 and Oncotype Dx [171] (see section 1.9.2).

1.9.1.5 Preoperative endocrine prognostic index

The Preoperative Endocrine Prognostic Index (PEPI) for recurrence free survival was developed from the P024 trial and validated in the IMPACT cohort. PEPI is calculated from tumour size, nodal stage, ER and Ki67 of the surgical specimen following neoadjuvant endocrine treatment, giving a score of 0 (good prognosis), 1-3 (intermediate) and 4+ (poor prognosis) which correlated to recurrence free survival (log rank p=0.002) [172]. PEPI 0 patients with early stage tumours had no recurrence events in 5 years in the training cohort, highlighting an excellent prognostic group who could forego adjuvant chemotherapy.

1.9.2 Prognostic gene signatures

1.9.2.1 Genomic Grade Index

Genomic Grade Index (GGI) is a 97 gene assay which can characterise tumour grade into low or high risk, which is superior to the Elston and Ellis pathological grading 1-3, in predicting recurrence free survival [173].

1.9.2.2 Oncotype Dx

Oncotype Dx uses quantitative real time polymerase chain reaction (qRT-PCR) measures of 21 genes (16 cancer related, 5 reference), to calculate a recurrence score which has been shown to correlate to survival, independent of age and tumour size, in node negative, tamoxifen treated ER+ breast cancer [174]. The recurrence score also predicts magnitude of

chemotherapy benefit in these patients. Those with a high score (>31) derive large benefit, and those with a low score (<18) derive minimal benefit only [175]. The Trial Assigning Individualised Options for Treatment (TAILORx) of 10,253 women, validated the use of a low recurrence score (revised to <10) to identify patients who could be spared chemotherapy but who would have been recommended to receive it based on clinicopathological features. Of the 1,626 patients with a low risk score, all were treated with adjuvant endocrine therapy, and only 88 events of recurrence or death occurred by 5 years [176]. More work is required to establish best management of women with an intermediate score (11-25).

1.9.2.3 PAM50ROR

PAM50 is another qRT-PCR assay, which employs 50 genes to categorise patients into one of the intrinsic molecular subtypes; luminal A, luminal B, HER2-enriched or basal, based on relative distance to the centroid of each subtype [177]. PAM50 combined with a 'risk of recurrence' (ROR) score, correlates to long term outcome and is also predictive of response to neoadjuvant chemotherapy. ROR is calculated using a formula which includes Pearson correlations to gene expression profiles of each of the intrinsic subtypes, combined with a proliferation score (mean expression of 18 gene sub-set of the PAM50 genes), and pathologic tumour size, producing a result ranging 0-100. ROR is prognostic independent of standard clinicopathological variables, however combination of both is overall superior [178] in predicting late distant recurrence after adjuvant endocrine therapy. ROR can identify low risk ER+ node negative tumours with >95% 10-year survival without chemotherapy; which is comparable to performance of Oncotype Dx [179]. PAM50ROR is available commercially as Prosigna.

1.9.2.4 MammaPrint

MammaPrint was developed from empirical microarray analysis of 78 breast cancer patients who were aged less than 55 years, with tumours 5cm diameter or less and node negative, who were deemed to have poor prognosis if they developed metastases within 5 years, and good prognosis if not. Supervised analysis of 25,000 genes identified 70 genes which could accurately predict prognosis. Genes involved in cell cycle, invasion and metastases, angiogenesis, and signal transduction were enriched in the poor prognostic profile, including cyclin E2, *MCM6*, *MMP9*, *MMP1*, *RAB6B*, *ESM1* and VEGF receptor *FLT1* [180]. The 70-gene signature was subsequently validated in a larger cohort of 295 patients, where a poor prognosis signature corresponded to 10-year survival of 54.6% +/-4.4, compared with 94.5% +/-2.6 with the good prognosis signature. Similarly, recurrence free survival was 50.6% +/-

4.5, and 85.2% +/- 4.3, in poor and good prognostic groups respectively [181]. MammaPrint has been externally validated by other groups in both node negative [182] and node positive patients [183], including a subgroup of 101 patients with 1-3 positive lymph nodes, who did not receive chemotherapy, where Mammaprint was superior to Adjuvant! Online at predicting breast cancer specific survival. The poor prognostic signature is also predictive of patients who will derive benefit from adjuvant chemotherapy in addition to endocrine therapy [184].

The microarRAy prognoSTics in breast cancER (RASTER) study prospectively assessed the feasibility of implementing MammaPrint into treatment. Risk as assessed by clinicopathological features, often differed from MammaPrint, with discordance in 37% with Adjuvant! Online, 39% with St Gallen guidelines, and 27% with Nottingham Prognostic Index; more often MammaPrint classifying as low risk, where clinicopathological features classified as high risk [185]. A follow up study at 5 years found in 219 MammaPrint low risk patients, disease free survival was 97%; and in 208 MammaPrint high risk was 91.7% (p=0.03). This is despite 15% (33/219) of low risk patients receiving adjuvant chemotherapy, vs 81% (169/208) of high risk patients [186], suggesting omission of chemotherapy in those with a low risk Mammaprint signature does not compromise outcome.

Microarray In Node negative and 1-3 positive lymph node Disease may Avoid ChemoTherapy (MINDACT), is a randomised prospective trial of 6,693 women with invasive, early stage breast cancer, comparing the 70-gene signature with Adjuvant! Online in identifying patients unlikely to benefit from adjuvant chemotherapy. Patients with low risk disease both genomically and clinically, had no adjuvant chemotherapy; those deemed high risk by both genomic and clinical factors did have chemotherapy. Those with discrepancy between clinical and genomic risk were randomised to undergo chemotherapy or not. High-clinical low-genomic risk patients, who did not receive chemotherapy (n=748), had a distant metastases free survival rate of 94.4% (95% CI 92.3-95.9) at 5 years. This is only 1.5 percent lower than the same risk patients (n=749) who did receive chemotherapy (95.9%, CI 94.0-97.2) [187]. Interestingly, of these high-clinical low-genomic risk patients, 48% had positive lymph nodes, 93% grade 2-3 disease, and 34% were aged 50 years or younger; all factors traditionally considered poorly prognostic. In patients deemed high-clinical risk with one positive node (n=801), or two or three positive nodes (n=405); the 70-gene signature indicated low risk disease in 505 (63%) and 226 (55.8%) respectively. The study was however underpowered to determine benefit of chemotherapy in those with discordant clinical and genomic risk, and needs longer term follow up in due course.

1.9.2.5 Endopredict / EndopredictClin

Endopredict (EP) combines three proliferation associated genes, five ER related genes and three housekeeping genes, assessed by qRT-PCR, to provide a score 0 to 15, to predict distant recurrence in endocrine treated ER+ HER2- patients [188]. EPclin combines EP with nodal status and tumour size into an algorithm to direct treatment decisions. EP and EPClin have been shown to provide significant prognostic information above that of Oncotype Dx in 928 patients with ER+ disease treated with 5 years of endocrine therapy; especially in years 5-10 of follow up and in patients node positive [189].

1.9.2.6 Breast Cancer Index

Breast cancer index (BCI) combines two prognostic gene signatures: molecular grade index (MGI) and *HOXB13:IL17BR* (H:I) ratio, to estimate risk of recurrence in node negative ER+ patients. MGI is a five-gene prognostic signature which calculates a genomic grade, and improves on histological prognosis of grade 2 tumours by classifying them as 'grade 1-like' or 'grade 3-like.' H:I ratio is a two gene marker of risk of recurrence in ER+ breast cancer treated with tamoxifen [190]. The combination outperforms either two independently, and also better stratifies risk of recurrence within 10 years than standard clinicopathological variables. BCI categorises patients as low, intermediate or high risk; which correlates to 10-year recurrence free survival of 87.5%, 83.9% and 74.7% respectively, in a validation cohort of 146 patients with ER+ disease, across both lymph node negative and positive subgroups [191].

1.9.3 Predictive Gene signatures

The Sensitivity to Endocrine Therapy (SET) Index was derived from a cohort of 437 ER+ cancers and is based on expression of *ESR1*-related genes, 106 with positive correlation and 59 with negative correlation. It categorises into high, intermediate and low risk groups which correlate to recurrence free and overall survival at 10 years in tamoxifen treated patients, but not in non-treated; suggesting it is predictive of response to tamoxifen [192]. Lymph node status was however also independently prognostic in the training cohort.

The ACOSOG Z1031B study comparing neoadjuvant letrozole, anastrozole and exemestane, assessed an on-treatment biopsy after 2-4 weeks. If Ki67 was >10% patients were deemed to be non-responsive to AI and switched to neoadjuvant chemotherapy or proceeded to surgery. 49 of 236 (20.7%) women had Ki67>10% at 2 weeks, of these 35 switched to chemotherapy, and pathological complete response was achieved in 2 patients. At 4.4 years follow up they also had a significantly increased risk of relapsed disease (log rank p=0.004) [193]. Gene

expression assessed by MultiGene Proliferation Score (MGPS) RNA assay demonstrated even in those with Ki67>10% at 2 weeks, the non-responders, there was still down regulation of MGPS, although to a lesser degree than in those with Ki67 <10%. One limitation of this study is the use of pCR to assess response to chemotherapy in ER+ tumours, which may explain the low rate demonstrated. Further survival data is also awaited.

A 4-gene signature, able to predict response to neoadjuvant AI based on expression of two genes pretreatment, namely *IL6ST* (associated with immune signaling) and *NGFRAP1*(apoptosis induction related), and two proliferation genes (*ASPM* and *MCM4*) after two weeks of letrozole, has been identified. This model has a 96% accuracy (96% sensitivity, 94% specificity; positive predictive value 98%, negative predictive value 89%) [149]. Blinded independent validation in a second cohort treated with neoadjuvant anastrozole yielded similar results, predicting response correctly in 40 of 44 patients; 91% accuracy, 90% sensitivity, 92% specificity. The 4-gene signature also correlates with progression free and breast cancer specific survival in the training cohort and can be assessed by qRT-PCR and/or IHC.

These studies confirm the added value of an on-treatment biopsy in predicting response to neoadjuvant endocrine therapy, and indeed long term outcome. There are several other ongoing trials looking to prospectively explore this further, using on-treatment biopsy assessment of response to alter decisions about ongoing treatment.

The trial of Peri-Operative Endocrine Therapy: Individualising Care (POETIC) is a phase III randomised prospective multicentre trial which recruited 4,486 patients over 5 years, comparing 4-weeks perioperative AI (2 weeks pre- and 2 weeks post-operative) with no therapy. The primary endpoint is to assess outcome in the perioperative AI group over surgery followed by standard adjuvant therapy group. A secondary outcome is to determine the most effective time points for molecular profiling, comparing measures of Ki-67 at baseline and at 2 weeks, in prediction of recurrence free and overall survival [194]. Recruitment closed in 2014 and patients are being followed up annually for 10 years.

The ALTERNATE trial is a further prospective trial currently recruiting to compare neoadjuvant anastrozole or fulvestrant or combination of both, for 6 months. After 4-weeks, Ki67 will be assessed, and patients switched to neoadjuvant chemotherapy if >10%. Patients will continue the same endocrine drug for adjuvant therapy and PEPI will also be assessed. The primary objective is to determine whether endocrine resistance is less with fulvestrant, or

fulvestrant plus anastrozole, than anastrozole alone. Secondary aims will examine degree of Ki67 suppression amongst others [195].

The NeoMONARCH phase II randomised multicentre study of 224 patients compared abemaciclib (CDK4/6 inhibitor, see section 1.10.5) alone, abemaciclib plus anastrozole, or anastrozole alone for 2 weeks preoperatively. Ki67 assessed at baseline and again at 2-week biopsy, was significantly more supressed at a 9-month interim analysis with abemaciclib either as monotherapy or in combination ($p<0.001$, $n=64$) than with anastrozole alone [196]. Again, we await survival outcomes in these patients.

Most of the above described prognostic gene signatures and clinicopathological criteria aim to distinguish ER+ patients with favourable prognosis from those with adverse prognosis. In HER2+ and TNBC treatment options are not so debated. Uncertainty arises in luminal disease, and particularly where it is safe to omit adjuvant chemotherapy to avoid overtreatment and its associated toxicities. Tumour biology and prognosis at genomic and transcriptomic level does not always correlate with traditional clinicopathological features. Indeed, many studies have alluded to the fact that combining biomarkers with clinical features may provide the most useful prognostic information [178, 179, 189], but there is by no means an adopted standard as yet.

1.10 Endocrine resistance

Although 70% of all breast cancers express ER, resistance to endocrine therapies is common. This can be *de novo* or intrinsic resistance present from the outset of disease, or it can be acquired in response to treatment, where a tumour initially responsive to treatment then progresses or recurs later. Response in the neoadjuvant setting is around 70% [147], however in the adjuvant setting up to 50% of patients will eventually develop resistance to one or more drugs [197]. The mechanisms of resistance are multiple. There is tumour heterogeneity in breast cancer, and tumours with a small number of ER+ cells amongst predominantly ER- cells have poorer and shorter response to endocrine treatment [198]. In such cases ER- clones would dominate over time and these tumours may exhibit *de novo* resistance despite containing some responsive cells, as the clinically appreciable change in the tumour would be negligible. Loss of ER expression, where a cancer initially all ER+ becomes ER- is one mechanism in a small number of patients, around 10% [172]. The majority however remain ER+ despite no longer responding to endocrine therapies, suggesting the tumour is able to progress independently of a functioning activated ER. Partial response to second or third line

agents is seen in those who retain ER expression [199]. This may be driven by cross talk between ER and alternative growth factors, most commonly the receptor tyrosine kinase human epidermal growth factor receptors (EGFR), HER1, HER2, HER3 and HER4. ER independent activation of the receptor and its subsequent downstream signalling is also causative.

Defining primary and acquired resistance has been problematic. Adjuvant endocrine therapy is given to ER+ disease in the hope of preventing micrometastatic disease causing a relapse. Patients who have an early relapse are likely to have primary endocrine resistance, however those who relapse later may still have endocrine sensitivity, and therefore may benefit from further ER+ targeted drugs, despite previous endocrine therapies. The Advanced Breast Cancer (ABC3) consensus group has recently therefore proposed definitions as follows:
Primary resistance – relapse within first two years of adjuvant endocrine therapy, or progressive disease within first 6 months of first line endocrine therapy in metastatic breast cancer.
Acquired resistance – relapse whilst on adjuvant endocrine therapy but after first two years, or relapse within twelve months of completing adjuvant endocrine therapy; or progressive disease more than 6 months after initiating endocrine therapy for metastatic breast cancer, whilst on endocrine therapy [200].

Mechanisms of endocrine resistance are multiple and complex, and may relate to host factors affecting bioavailability and drug adherence, and also molecular mechanisms affecting the tumour microenvironment and the tumour cell and oncogenic signalling [201]. Herein are discussed the most common mechanisms of endocrine resistance, and current and emerging targeted drug therapies against them, summarised figure 1-7.

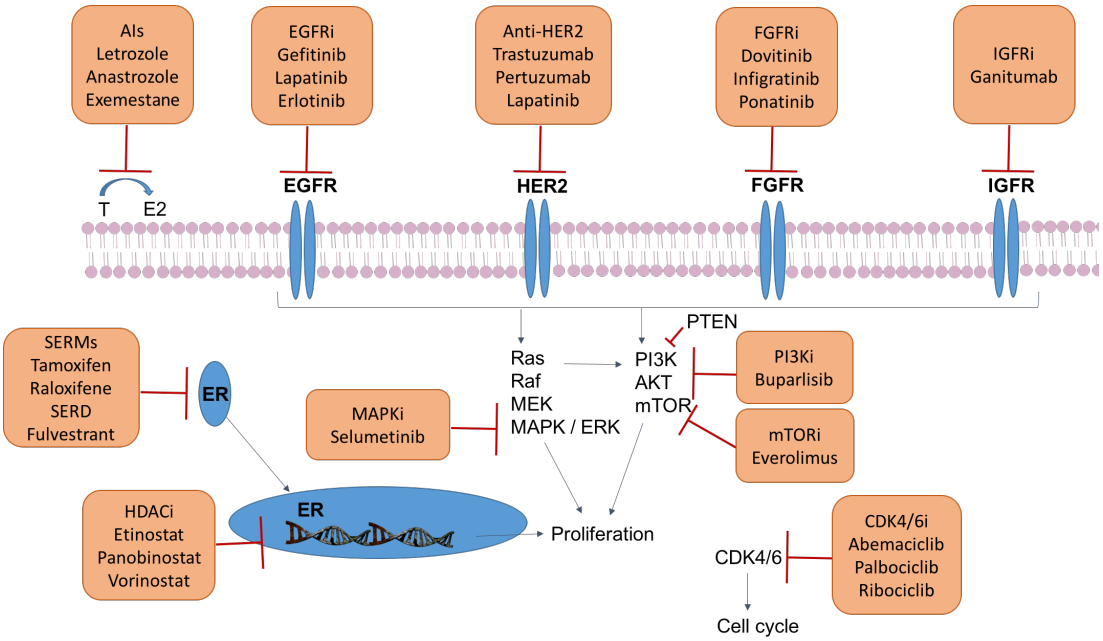

Figure 1-7. Mechanisms of endocrine resistance and endocrine therapy targets
Schematic diagram of receptors and pathways commonly involved in endocrine resistance, adapted from Selli et al 2016 [202].
Current and novel drugs in orange squares
CDK - cyclin dependent kinase, EGFR - epidermal growth factor receptor,
FGFR - fibroblast growth factor receptor, HDAC - histone deacetylase i - inhibitor,
IGFR - insulin-like growth factor receptor, MAPK - mitogen activated protein kinase,
mTOR - mammalian target of rapamycin, PI3K - phosphatidylinositol-3kinase,
PTEN – phosphatase and tensin homolog

1.10.1 ER and Growth Factors

Multiple receptor tyrosine kinases (RTKs) including the EGFRs [203], fibroblast growth factor receptors (FGFR) [204] and insulin-like growth factor receptors (IGFR) [205] have been implicated in endocrine resistance, by ligand independent activation of ER. This may be achieved by downstream signalling through different kinase pathways including PI3K and MAPK [206] that can activate ER proteins by phosphorylation.

In ER+ cell lines increased levels of EGFR and HER2 have been demonstrated at time of tamoxifen resistance [206]. This led to several studies assessing the effect of dual blockade of EGFR plus ER, regardless of HER2 expression, although these have failed to show any advantage of this approach. Gefitinib, an EGFR inhibitor, combined with tamoxifen had no

significant advantage in PFS over placebo in ER+ metastatic disease [207]. Ganitumab, a monoclonal IGF-1R antibody, in addition to endocrine therapy did not improve outcome in locally advanced and metastatic ER+ breast cancer [208]. Lapatinib combined with letrozole failed to improve PFS in ER+HER2- metastatic patients [209]. It seems reasonable to conclude that targeting growth factor receptors when it is unknown which one is driving the process of endocrine resistance is ineffective.

1.10.1.1 ER and HER2

ER+HER2+ tumours account for around 10% of all breast cancers, and are less responsive to endocrine therapy than ER+HER2- tumours [210] [211]. Dual targeting of ER and HER2 has been effective. In metastatic ER+HER2+ breast cancer the addition of trastuzumab to anastrozole improved progression free survival (PFS) from 2.4 to 4.8 months compared with anastrozole alone [212]. Also in the metastatic setting, the combination of letrozole and trastuzumab was shown to produce a sustained clinical benefit of at least 1 year in most patients [213]. Lapatinib, a dual tyrosine kinase inhibitor of EGFR and HER2, given in addition to letrozole increased PFS from 3 to 8.2 months compared with placebo in metastatic ER+HER2+ patients [214].

1.10.2 Oncogenic pathway activation

The phosphatidylinositol 3-kinase (PI3K) / AKT / mTOR pathway is an oncogenic signalling pathway frequently implicated in endocrine resistant breast cancer, (see also section 3.1). PI3K can be activated by growth factor receptor tyrosine kinases and G-protein coupled receptors. Mutation or amplification of the genes encoding the PI3K catalytic subunits, p110α (*PIK3CA*) and p110β (*PIK3CB*), also cause activation. PI3K phosphorylates phosphatidylinositol 4,5-bisphosphate (PIP_2) to phosphatidylinositol 3,4,5-trisphosphate (PIP_3) which recruits proteins including AKT and PDK1 to the plasma membrane, driving cell cycle survival and progression on activation [215]. AKT activates the mammalian target of rapamycin (mTOR)-containing complex 1 (TORC1) which regulates protein synthesis. Inhibition of the PI3K pathway is maintained by the lipid phosphatases, phosphatase and tensin homolog (PTEN) and INPP4B, which dephosphorylate PIP_3 and PIP_2 respectively [216].

Pre-clinical studies demonstrate enhanced anti-tumour effects with dual blockade of ER and PI3K pathways, which lead to the development of a trial of 4 months neoadjuvant letrozole plus everolimus, an mTOR inhibitor, versus letrozole plus placebo in 270 postmenopausal women with ER+ early breast cancer. Clinical response was higher in the everolimus arm

(68.1% vs 59.1%, p=0.62) as was reduction in Ki67 (57% vs 30%, p<0.01) [217]. The Breast Cancer Trials of Oral Everolimus-2 (BOLERO2) has confirmed benefit of this dual approach in metastatic ER+ disease, where everolimus restores sensitivity to exemestane in progressive disease. In 726 women, progression free survival increased to 6.9 months in exemestane plus everolimus, versus 2.8 months in exemestane plus placebo (HR 0.43, 95% CI 0.35-0.54, p<0.001) [218].

The role of pan-PI3K inhibitors, such as buparlisib, is also being explored. The BELLE-2 trial of 1,147 patients with metastatic AI resistant ER+ disease, found median progression free survival was 6.9 months with fulvestrant and buparlisib, versus 5 months with fulvestrant plus placebo (HR 0.78, p<0.001). Adverse events were also much more common in the combination group however (77.3% vs 32%) with consequent increased discontinuation rates (13.2% vs 1.8%). PFS was more enhanced in those with *PIK3CA* mutations in their archived original tumour with fulvestrant and buparlisib over fulvestrant and placebo (6.8 months vs 4 months, HR 0.76, p=0.14). 200 patients were found to have a *PIK3CA* mutation in circulating tumour DNA at enrolment and in this subgroup, median PFS was improved further than the overall cohort with buparlisib over placebo (7 months vs 3.2 months, HR 0.54, p<0.001) [219]. Full survival outcomes from this trial have not yet been reported.

The Mitogen Activated Kinase Pathway (MAPK) is a further signalling pathway that is activated by growth factor receptors and has been implicated in endocrine resistance. MAPK is stimulated by the RAF serine/threonine kinase and activates additional downstream kinases such as ERK, c-Jun and p38MAPKs which phosphorylate downstream transcription factors [201]. Trials dual targeting ER and MAPK are ongoing now. A phase II trial of fulvestrant alone or in combination with AZD6244, a MAPK inhibitor, in metastatic AI resistant ER+ cancer finished recruiting in September 2016 [clinicaltrials.gov NCT01160718].

1.10.3 *ESR1* mutations

Mutations in the *ESR1* gene encoding ERα are present in 21% of metastatic ER+ breast cancers [220]. Mutations cluster in a small region encoding the ligand binding domain, most commonly at amino acids 537 and 538, causing ligand independent activation of ER with promotion of ER dependent genes *in vitro* [221]. Toy *et al* confirmed a mutation was present in 9 of 36 (25%) metastatic tumours. Two matched primary samples from diagnosis were available, neither of which had *ESR1* mutation. Using the BOLERO2 cohort of endocrine resistant metastatic breast cancers, *ESR1* mutations were found in 5 of 44 (11%) tumours; with

only 6 of 138 (3%) having ESR1 mutations at diagnosis. Further, Robinson *et al* described 6 of 11 (54.5%) metastatic endocrine treated patients with *ESR1* mutations, with matched diagnostic samples from 3 revealing no *ESR1* mutations [222]. In 962 samples at diagnosis in The Cancer Genome Atlas Network, *ESR1* mutations were only present in 0.5% [67]. Given most metastatic ER+ patients will have received endocrine therapy, we can assume *ESR1* mutation is acquired over time and in response to endocrine therapy. This contrasts with the frequency of other common breast cancer genomic alterations, with comparable rates in primary and metastases of *TP53*, *CCND1*, *MCL1*, *MYC*, *FGFR1* [223] and *PIK3CA*, see also chapter 3. Jeselsohn described *ESR1* mutations in 9 of 76 (12%) metastatic ER+ tumours; but this increased to 5 of 25 (20%) when selecting patients who had received multiple lines of prior endocrine therapy [223]. In the PALOMA3 trial (see section 1.10.5) *ESR1* mutations were found in 91 of 360 (25.3%) patients. In multivariate analysis, *ESR1* mutations were associated with previous aromatase inhibitor use (rather than tamoxifen), sensitivity to prior endocrine therapy, and bony or visceral metastases [224]. The Study of Faslodex with or without concomitant Arimidex vs Exemestane following progression on non-steroidal Aromatase inhibitors (SoFEA) trial, compared fulvestrant plus anastrozole, fulvestrant plus placebo, and exemestane in 723 ER+ patients with advanced or metastatic disease who had progressed or relapsed on a non-steroidal AI. Overall there were no significant differences in treatment arms in progression free or overall survival [225], however in retrospective analysis they identified *ESR1* mutations in 63 of 161 (39.1%) patients. In this subgroup was a significantly superior effect of fulvestrant over exemestane; with progression free survival 5.7-months (95% CI 3.0-8.5) vs 2.6 months (95% CI 2.4 – 6.2); (HR 0.52, 95% CI 0.30-0.92; p=0.02) [226].

1.10.4 ER co-regulators

Co-regulators are proteins that bind to ER and modify its effectiveness in controlling gene expression; by increasing (coactivators) or reducing (corepressors) its activity. Coactivators including AIB1 and MNAR/PELP1 [201] can become amplified causing hyperstimulation of ER leading to tumourigenesis and cancer progression. Corepressors including N-COR and SMRT, which decrease ER function, have also been implicated in endocrine resistance [86]. Another mechanism of facilitating transcription factor interaction with chromatin is through pioneer factors, transcription factors which directly bind to condensed chromatin independently of other proteins and can modulate chromatin structures. Pioneer factors recruit other transcription factors and histone modification enzymes, controlling DNA methylation. Forkhead protein (FOXA1) [227], TLE proteins, AP2Y and PBX1 have been proposed as

pioneer factors in breast cancer [228]. Pioneer factors do not require additional transcription factors therefore allow quick interaction between transcription factors and chromatin, resulting in rapid transcription.

1.10.5 Cell cycle signalling

Endocrine therapy leads to G1/S phase cell cycle arrest. Cyclins are crucial to cell cycle progression, and aberrant regulation of positive and negative regulators of the cell cycle, including MYC, cyclins-D1 and E1, p21 and p27 have been implicated in endocrine resistance [229]. Expression of cyclin-D1 is higher in luminal tumours than in other subtypes, particularly luminal B, whereas cyclins-B1 and E1 are more common in basal tumours [230]. Increased expression of cyclin-D1 is achieved as a direct transcription factor of ER, or by amplification of *CCND1*, or by mitogenic signalling pathways. Cyclin-D1 binds to cyclin dependent kinases 4 and 6 (CDK4/6) which partially phosphorylates the retinoblastoma (RB) tumour suppressor protein, losing its inhibitory function and activating S-phase transcription genes (including cyclin-E and CDK2) driving cell cycle progression [231].

CDK4/6 inhibitor drugs, such as palbociclib and ribociclib, have proven highly effective in prolonging benefit from endocrine therapy. The Mammary Oncology Assessment of LEE011's (Ribociclib's) Efficacy and Safety (MONALEESA-2) trial assessed ribociclib plus letrozole against placebo plus letrozole in 668 patients with advanced ER+ HER2- breast cancers with no prior endocrine therapy (except non-steroidal AI completed >12 months prior to recurrence). Ribociclib was significantly superior to placebo in median progression free survival, and in fact was not yet reached by the time of interim reporting (95% CI 19.3 months – not reached, vs 14.7 months, 95% CI 13-16.5) [232].

The PALbociclib: Ongoing trials in MAnagement of breast cancer (PALOMA)-2 trial compared letrozole plus palbociclib with letrozole plus placebo in 666 women with ER+ HER2- metastatic breast cancer. Progression free survival was significantly improved with palbociclib; 24.8 months (95% CI 22.1 months – not estimable) vs 14.5 months (12.9-17.1), (HR 0.58, 95% CI 0.46-0.72, $p<0.001$) [233].

The PALOMA3 trial assessed fulvestrant plus palbociclib over fulvestrant plus placebo, in 521 patients with metastatic ER+ breast cancer; all of whom had between one and three previous endocrine therapies. Progression free survival was significantly improved with

palbociclib; 9.5 months (95% CI 9.2-11.0) vs 4.6 months (3.5-5.6) (HR 0.46, 95% CI 0.36-0.59, p<0.0001) [224].

Toxicity, particularly neutropenia, is problematic with CDK4/6 inhibitors however, occurring in 66.4% of patients with ribociclib and 1.4% of placebo in MONALEESA2. In PALOMA3 80% of patients experienced neutropenia, and 54% had to have dosing interruptions consequently. The effect on overall survival is still to be established with CDK4/6 inhibitors also.

1.10.6 Epigenetics

Epigenetics refers to the reversible modification of DNA and gene expression without altering DNA sequence. DNA methylation, histone modification and nucleosome remodelling are major epigenetic mechanisms in breast cancer. DNA methyltransferases (DNMTs) interact with histone deacetylases (HDACs) and the methyl-CpG-binding domain (MBD) proteins to mediate DNA methylation. Epigenetic silencing of ER regulated genes and *ESR1* itself have been implicated in endocrine resistance [234]. The Cancer Genome Atlas identified five methylation subgroups with over 4,000 differentially methylated genes. One hypermethylated phenotype was significantly enriched with luminal B mRNA subtype tumours [67].

A small phase II trial of 43 patients found Vorinostat, a HDAC inhibitor, combined with tamoxifen was well tolerated in ER+ metastatic breast cancer patients who had disease progression on endocrine therapy. Clinical benefit, defined as response or static disease >24 weeks, was achieved in 40% [235]. Entinostat another HDAC inhibitor, was assessed in a phase II trial of 130 women with progressive disease on non-steroidal AI. Exemestane plus entinostat improved PFS to 4.3 months vs 2.3 months with exemestane plus placebo (HR 0.73, 95% CI 0.5-1.07, p=0.055) [236]. Luminal B tumours may benefit more from epigenetic drug targeting, given the higher frequency of methylation in this subtype.

1.10.7 Tumour heterogeneity

Breast cancer is inherently heterogeneous, a feature which enhances metastatic potential and resistance to drug therapies. Heterogeneity is regulated by genetic and epigenetic features, as well as tumour microenvironment. It is recognised that expression of ER, PR and HER2 between a primary breast cancer and its metastases is discordant in up to 40% [237] (see also section 5.1). Theories around establishment and maintenance of tumour heterogeneity include the cancer stem cell (CSC) and clonal evolution model theories. The CSC concept proposes a

hierarchy system whereby a minor subset of CSCs at the apex divides into multiple proliferating terminally differentiated cells at the base [238].

The clonal evolution model proposes that cancer cells within a tumour possess varying degrees of genetic instability and acquire more aberrations in tumour evolution over time and in response to treatments. A tumour is thereby composed of cells with shared founding genetic events, and additionally within the tumour, multiple subpopulation cell groups with specific individual genetic aberrations [239]. Over time further genetic instability generates more alterations and diversity, many of which are lost. However, if a change promotes growth or survival advantage, the clone harbouring this will become predominant [239], see figure 1.8. Mutations which have been implicated in drug resistance may therefore become the overall phenotype of the tumour [86] which may contribute to acquired endocrine resistance in tumours initially responsive to treatment.

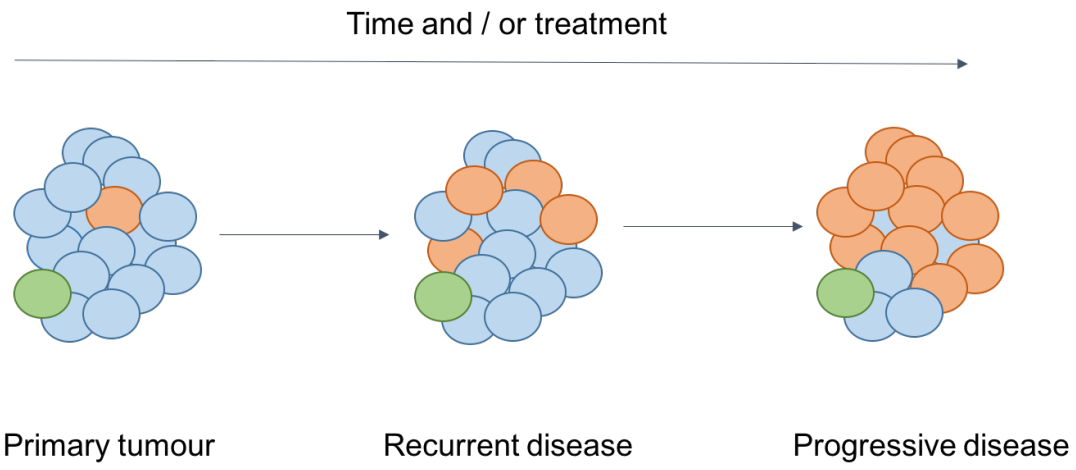

Figure 1-8. Subclonal diversification in breast cancer
Intra-tumour genetic heterogeneity is present but not predominant in primary tumour. Genetic aberration in orange and green cells.
Over time orange subclone becomes predominant and that genetic aberration will become overall phenotype of tumour.

1.10.7.1 Liquid biopsy

Circulating tumour cells (CTCs) are assessable in plasma in patients with metastatic BC. A trial of 1,944 patients from 20 studies found a lower CTC count at baseline and after 3-5 weeks of treatment was associated with improved progression free and overall survival [240]. CTCs can also undergo molecular profiling to assess for biomarkers of drug resistance. Circulating cell-free tumour DNA (ctDNA) can also be sampled from serum and has been suggested to be more sensitive than CTCs in predicting treatment response [241]. Standardised measures need to be implemented before this novel technology is routinely available. However, in the future it may allow monitoring for biomarkers of resistance in the metastatic setting. Additionally in the adjuvant setting where there is no solid tumour to biopsy, a liquid biopsy could allow serial monitoring of any residual tumour burden to identify patients at risk of early relapse by highlighting markers of endocrine resistance [202].

1.10.8 Overcoming endocrine resistance

In metastatic breast cancer, combination therapies tend to be more effective than monotherapy. There is no doubt that dual therapies can delay the onset of acquired resistance. Tumours which respond to first line endocrine agents, will likely respond to second and third line agents. However, the progression free interval will decrease at each stage [242]. ASCO have issued updated guidelines for the management of ER+ metastatic breast cancer [243]. An attempt to summarise these and the evidence from the trials discussed above is presented in the following flow diagram, see figure 1.9. This is however a broad oversimplification of this complex issue. Realistically in patients with *de novo* metastatic or endocrine naïve ER+ disease, first-line monotherapy with an AI is most likely. A sequential approach is preferred rather than dual therapy at presentation, whereby one agent is utilised until there is evidence of progression, then a second agent is adopted until progression, then a third and so on. A further problem is a lack of biomarkers to predict who will benefit from each of these targeted therapies. We must remember the toxicities associated with novel drugs and consider their impact on quality of life in this cohort.

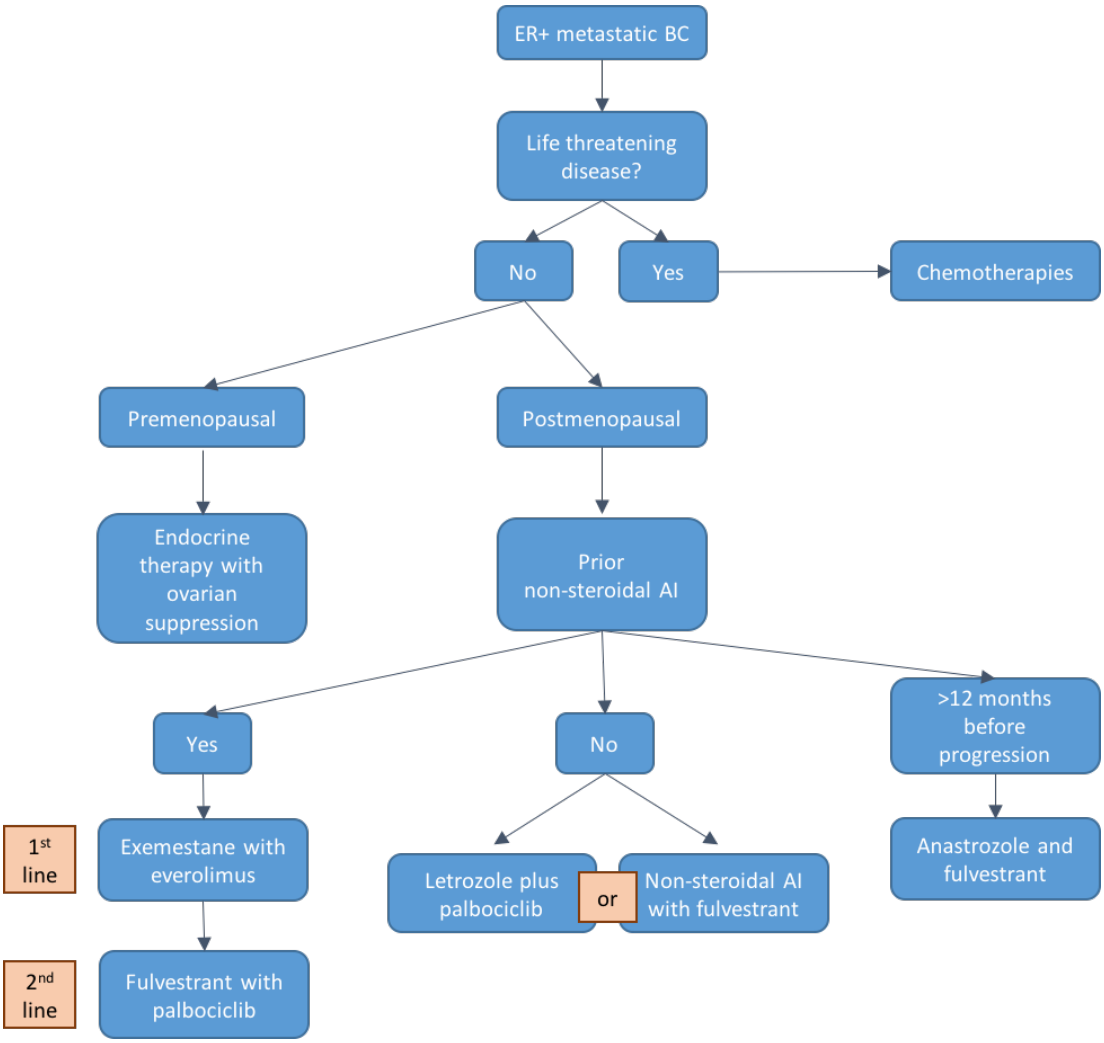

Figure 1-9. Treatment of metastatic ER+ breast cancer

BC – breast cancer, AI – aromatase inhibitor

1.11 Summary and approaches

The high throughput genomic technologies have revolutionised our understanding of breast cancer [36]. This complex disease is a spectrum with varying risks and clinical phenotypes. Multiple subtypes have been identified which correlate to prognosis [41]. Indeed, multiple methods of defining and identifying those subtypes have also been proposed.

As clinicians, our aim is to stratify risk through measures of prognosis, in order to best advise patients on treatment. High risk disease must of course be identified early, but equally important is to identify the very good prognosis tumours [172], and spare these patients from toxic over-treatment, which would offer them little benefit. Standard clinicopathological disease measures are effective in estimates of prognosis, but do not always consider the innate biology of the tumour, which could misrepresent risk in a significant proportion of patients [161]. Multiple gene signatures predictive of recurrence and outcome have been proposed [149, 161, 169, 170, 172, 175, 177, 188], and the optimal signature is certainly still debated. Some promise has been demonstrated through combining variables from traditional clinicopathological features with molecular biomarkers [178, 189], and this is likely to prove to be the best option in due course.

ER+ breast cancer accounts for 75% of the disease. Endocrine treatment is the mainstay of therapies in these patients. In premenopausal women, tamoxifen [86] plus ovarian suppression in high risk or young patients [113], has been established as best care. In postmenopausal women AIs are superior [118, 119]. Recent advances include extended duration of adjuvant therapy, and in the metastatic setting, sequential endocrine treatments [243], which offers a real option of delaying acquired resistance and disease progression.

The rate of resistance to endocrine therapy approaches 50% in the adjuvant setting. The mechanisms by which this is achieved are complex and not fully understood. Pathways involved commonly include EGFR, HER2, MAPK and PI3K [201]. Genomic aberrations in *ESR1* and *PIK3CA* [67], amongst other genes, have been frequently implicated in luminal disease. It is possible that multiple varied mechanisms of resistance are active in individual patients at different time points in the history of their disease. Recent large phase III randomised controlled trials have proven the benefit of combining standard endocrine agents with newer drugs, including fulvestrant with AI [110], CDK4/6 inhibitors with AI [233], and mTOR inhibitors with AI [218].

The neoadjuvant period offers a unique possibility, through sequential biopsy, to explore molecular changes in response to drugs, to explore prognostic and predictive biomarkers, and the opportunity to predict long term outcomes very early in the course of disease. Drugs which have been validated in the metastatic setting are now increasingly being applied in the neoadjuvant setting also [196]. However, we currently do not have biomarkers to predict which patients will benefit from particular targeted agents, and this represents a major clinical need.

1.12 Aims of Thesis

The four projects incorporated in this thesis aim to explore ER+ breast cancer and its evolution over time, treatment and progression. Sequential matched patient biopsies are utilised to reduce the variability associated with sampling and processing, and therefore increase the statistical power of any relevant findings. Gene expression profiling will evaluate molecular changes in the presence and absence of endocrine treatment and in lymph node metastases. Changes related to histological subtype will be explored also. The following research aims to establish:

- Comparisons of the gene expression profiles of breast cancer in the absence of treatment in the normal time course of the disease between diagnosis and resection.
- Effect of time between biopsies on any changes identified in tumour biology and any relationship to clinical outcome.
- A large negative control cohort to be used as a comparison for any future studies exploring biomarkers in the neoadjuvant setting.
- Comparison of gene expression profiles of the clinicopathologically distinct breast cancer subtypes; invasive lobular and invasive ductal carcinoma.
- Changes in gene expression profiles in response to neoadjuvant letrozole in lobular and ductal subtypes.
- Correlation of *PIK3CA* mutation and PTEN status in primaries and patient-matched recurrences and metastatic samples.
- Relationship of *PIK3CA* and PTEN status at diagnosis to long term outcome.
- Comparison of gene expression profiles of matched primary breast tumour and synchronous lymph node metastasis.
- Relationship of tumour biology of breast primary and metastatic lymph node to clinical outcome.

2 Molecular effects of initial core biopsies in neoadjuvant window studies in breast cancer

2.1 Introduction

Endocrine therapy is effective in most invasive ER+ breast cancers however some tumours demonstrate innate resistance. There is a clinical need to identify these patients early, in order they be switched to alternative or combination therapies. The value afforded by the neoadjuvant window of opportunity treatment period is unquestionable. Measures of response to therapies and long term survival are possible after only short courses of treatment [30, 152]. The ACOSOG Z1031B trial recently reported poorer long-term outcomes in patients with higher Ki67 after 2 weeks of neoadjuvant aromatase inhibition [193]. Further trials are ongoing to validate the use of on-treatment biopsies as predictive and prognostic markers. These include ALTERNATE, where on-treatment Ki67 will determine whether to continue neoadjuvant endocrine therapy, or to switch to surgery or chemotherapy instead [195]; and NeoMONARCH, where on treatment Ki67 will be used to assess prognosis relative to abemaciclib, a drug relatively new to the neoadjuvant setting. Recently we identified a 4-gene signature which can predict response or resistance to neoadjuvant letrozole with 96% accuracy, based on expression of 2 genes at baseline, *IL6ST* (immune related) and *NGFRAP1* (apoptosis); and a further 2 genes after 2 weeks of treatment, *ASPM* and *MCM4* (proliferation related) [149]. After a breast cancer diagnosis, there is an inevitable time delay before surgery in the order of a few weeks, to complete staging investigations and to agree on appropriate surgical approaches. Some argue that in ER+ disease (a result which will be available from the core biopsy within 24 hours usually), patients should be immediately started on endocrine therapy. Not only is this possibly associated with better long-term outcome [137], it also allows a valuable assessment of response in order that appropriate adjuvant treatment discussions can arise. The POETIC trial aims to assess this theory. It seems plausible that peri-operative treatment and sequential biopsy from the same tumour is going to become increasingly routine in breast cancer treatment [244].

There are challenges to this approach however. There is innate biological heterogeneity within the primary tumour [245] and with synchronous metastases [237]. Also, the ideal interval at which to assess response with a second biopsy is debated. Miller *et al* previously demonstrated a significantly higher proportion of genes changed at 3 months in response to neoadjuvant letrozole compared to 2 week biopsies in the same patient [246]. Chen *et al* compared the effect of time interval between core biopsy and surgical resection on the expression of ER,

PR, HER2 and Ki67 in 276 patients. They found good concordance for ER (94.2%), PR (87%) and HER2 (97.1%) but found Ki67 was significantly higher in the surgical specimen (29.1%) vs the core biopsy (26.2%), $p<0.001$, an effect which was significantly associated with length of time between the 2 biopsies ($p=0.01$) [247]. We note their inter-biopsy interval was very short, comparing groups by 1-2 days, 3-4 days or 5 days or more. It is feasible the changes could be even greater therefore, over longer time. It is rare for patients to have surgery so soon after core biopsy, therefore a longer time interval for biopsy related effect, or simply tumour evolution, should be explored. In a similar study Meattini *et al* compared IHC at core biopsy and surgical specimen, and found concordance of ER in 94.1%, PR in 88.1% and Ki67 in 88.1%. Using a 15% cut off to differentiate between high and low Ki67, they applied the St Gallen recommendations to determine molecular subtype at both biopsies, and found concordance of 87.1% [248]. Although this reflects good reproducibility, they highlighted a subgroup of patients where again Ki67 increased at second biopsy; and in 7 patients this re-classified them as luminal B rather than luminal A. This obviously has major implications for adjuvant treatment in these patients.

Haded *et al* compared gene expression profiles by a breast cancer disease specific array, of 12 patients at core biopsy and surgery. Pathways involving cellular metabolism and immune system were upregulated at second biopsy; and rho, integrin and ER pathways became down-regulated, despite having no intervening treatment [249].

Even the biopsy itself has been proposed as having an effect on the molecular evolution of a tumour. Morrogh *et al* demonstrated that 10 days exposure to neoadjuvant anastrozole produced significant changes in cancer related and ER regulated genes, which were not observed in a control group in their cohort of 31 patients [250]. They demonstrated changes in only 2 genes common to both treated and non-treated groups, *FOSB* and *MLL*, both involved in transcription, and proposed a wound healing response to the biopsy itself. This occurred despite ensuring the second biopsy was taken away from the site of the initial one.

Although there are increasing clinical trials assessing IHC at different time points *in vivo*, there are fewer available assessing at molecular level changes in response to treatment in the same patient [251]. Comparing matched pre and post treatment samples from the same patient removes much variability, but attempts to characterise patient-matched samples following neoadjuvant endocrine therapy or chemotherapy previously have often been limited to

relatively small numbers of patients [252-256] which markedly restricts the power implication of any significant findings.

In order that we can be certain that a drug effect observed in the neoadjuvant period is indeed due to that drug, a control cohort is crucial. This study incorporates one of the largest cohorts of patient matched core biopsy and surgical resection specimens with no intervening treatment, assessing gene expression profiles at both time points, in order to assess what if any, processes alter in the absence of treatment. We also aim to assess the effect of time interval between both biopsies and corresponding gene changes, exploring normal tumour evolution over time.

2.2 Methods

2.2.1 Patients

Paired fresh frozen tissue specimens from an initial diagnostic core biopsy and a second biopsy (median 33 days later, range 4-106 days), were identified from 83 patients treated for a primary histologically confirmed invasive breast cancer at the Western General Hospital, Edinburgh, between 2003 and 2011. All patients gave informed consent to be included in the study, which was approved by the Lothian Regional Ethics Committee (2001/8/80 and 2001/8/81). From these a cohort of 61 patients with paired samples was selected for further analysis based on ER status (all patients were ER-rich with Allred scores of 6, 7 or 8 as assessed by IHC by a dedicated breast pathologist). The study consisted of two independent arms: 36 patients treated with neoadjuvant letrozole with good clinical response after 3 months (data published previously [149]); and 25 patients who had no intervening treatment prior to surgery (figure 2-1).

Clinical response in the letrozole treated group was assessed by periodic measurement of tumour volume using three-dimensional breast ultrasound by a single operator (JMD); and defined as reduction in tumour volume of 70% by 3 months of treatment. Patient and tumour characteristics were recorded from paper and electronic case note review.

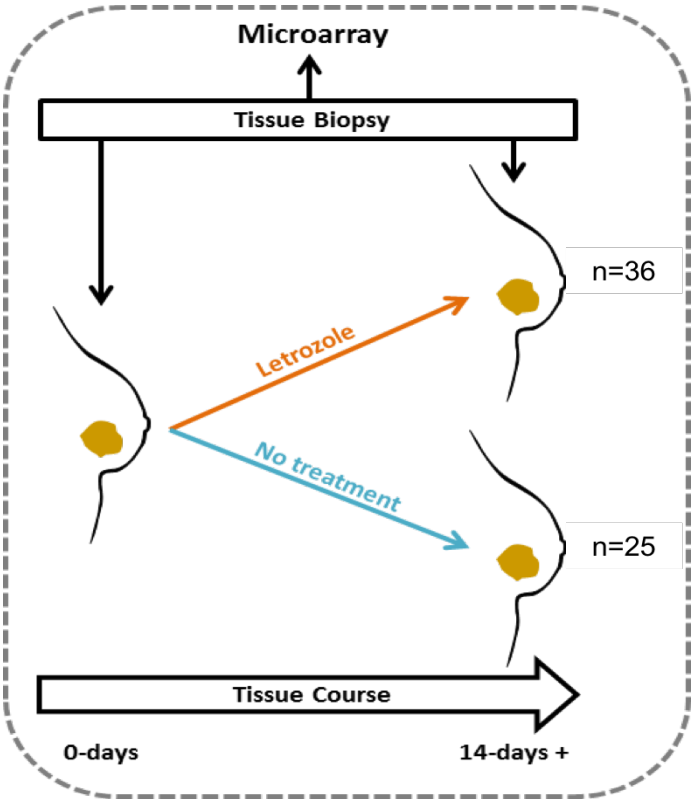

Figure 2-1. No intervening treatment, study design

2.2.2 Samples

Core biopsies were taken at diagnosis in all patients using a 14-guage needle device. In the letrozole-treated group the second biopsy was a repeat core biopsy taken after a median 17.5-days of treatment (range 4-106 days). In the letrozole treated group 32 of 36 (88.9%) patients went on to have surgery after 3 months of neoadjuvant letrozole. In the no-intervening treatment arm, biopsies were taken at diagnosis and again at surgery from the resection specimen at a median of 23 days later (range 14-35 days). Samples were snap frozen in liquid nitrogen and stored at -80˚C for later use.

2.2.3 RNA processing and microarray hybridisation

Tissues were homogenised and RNA extracted using the RNeasy Mini Kit with RNAse Free DNAse treatment (Qiagen). RNA quantity and quality was assured using a Nanodrop 2000c spectrophotometer (Thermo Scientific). RNA was reverse transcribed and amplified using the WT-Ovation FFPE System Version 2 (NuGEN), purified using the Qiaquick PCR purification Kit (NuGEN), biotinylated using the IL Encore Biotin Module (NuGEN), and purified using the minElute Reaction Cleanup Kit (Qiagen). At each step RNA/cDNA quantity and quality

was assured by repeat assessment with the Nanodrop 2000c prior to advancing to the next stage. Labelled cDNA was hybridized to Illumina Human HT-12v4 whole-genome expression bead arrays according to the standard protocol for NuGEN labelled samples. Data was extracted using the GenomeStudio software (Illumina).

2.2.4 Data Analysis

Illumina data from both arms of the study were independently pre-processed and re-annotated to Ensembl gene identifiers, then combined with cross-platform integration approaches to correct for batch effects as described previously [257]. Briefly, Illumina probe profiles were quantile normalized using the lumi package and mapped to Ensembl gene sequences using reMOAT [258], BioMart [259], and a custom BLAST sequence search. The datasets were filtered using detection p-values, removing probes that were undetected ($p>0.05$). Both datasets were then combined and batch corrected with cross-platform normalization. Pairwise and grouped differential gene expression analyses was performed using rank products (RP) (MeV; TM4 Microarray Software Suite). Functional gene ontology analysis was performed using DAVID Bioinformatics Resources 6.7 and the PANTHER Classification System. Gene expression heatmaps were generated in MeV using Euclidean distance with complete linkage following gene mean-centering performed in Cluster 3.0.

Clinicopathological features at diagnosis were compared using analysis of variance (ANOVA) for patient age and tumour size, and all other features using Fisher's exact test; or unpaired t-test, following construction of contingency tables. All statistics were performed in GraphPad Prism version 6.00 for Windows, (GraphPad Software, La Jolla California USA).

2.3 Results

The treated and non-treated groups were well matched, with no significant differences observed for histology, tumour grade, nodal stage, ER or HER2 positivity. In the letrozole treated group there were more T4 tumours and in the non-treated group there were more T1 tumours. This likely reflects a clinical decision to treat larger and locally advanced tumours with neoadjuvant letrozole in the hope of achieving breast conservation surgery at a later date. The groups were also well matched with regards to time between the two biopsy samples, with a median of 23 and 17.5 days in the no intervening treatment and letrozole treated groups respectively (table 2-1).

Variable	No treatment		Letrozole treated		
	n 25	(%) (100)	n 36	(%) (100)	P value
Histology					
Ductal	19	(76)	28	(77.8)	P=0.26
Lobular	6	(24)	4	(11.1)	
Unknown	0		4	(11.1)	
Tumour stage					
T1	11	(44)	5	(13.9)	**P=0.03**
T2	13	(52)	12	(33.3)	P=0.37
T3	1	(4)	4	(11.1)	P=0.26
T4	0		9	(25)	**P=0.003**
Tx	0		6	(16.7)	
Nodal stage					
N0	18	(72)	17	(47.2)	P=0.3
N1	5	(20)	12	(33.3)	
N2	2	(8)	0		
Nx	0		7	(19.4)	
Grade					
Grade 1	2	(8)	1	(2.8)	P=0.51
Grade 2	16	(64)	7	(19.4)	
Grade 3	7	(28)	5	(13.9)	
Unknown	0		23	(63.9)	
ER Allred Score					
6	1	(4)	1	(2.8)	P=0.59
7	3	(12)	7	(19.4)	
8	21	(84)	23	(63.9)	
Unknown	0		5	(13.9)	
Her2					
Positive	5	(20)	5	(13.9)	P=1
Negative	20	(80)	20	(55.6)	
Unknown	0		11	(30.1)	
	Biopsy interval(days)		Days on treatment		
Mean	23.8		23.7		
Median	23		17.5		
SD	6.3		20.6		
Range	14-35		4-106		

Table 2-1. Treated and non-treated cohort and tumour characteristics.

2.3.1 Gene changes in treated and non-treated tumours

In the letrozole treated group differential gene expression analysis (pairwise RP, FDR 0.01) identified 591 genes consistently up-regulated, and 380 down-regulated in response to treatment, figure 2-2. Gene ontology found these genes to be functionally enriched for up-regulation of the immune system and processes (including *CXCL9*, *C1QB*, *C3*, *C7*, *CCL19*, *ACKR1*), and extra-cellular matrix (ECM) remodelling (including *COL1A1*, *COL5A2*, *FBLN5*, *ELN*, *ECM2*, *SPP1*). Down-regulated genes were associated with proliferation, not unexpectedly (including *AURKA*, *CCND1*, *CCNB1*, *CKS2*, *MCM2*, *CENPN*, *CHEK1*, *TOP2A*, *UBE2C*). In the non-treated group 475 genes were consistently up-regulated and were functionally associated with immune processes (including *ITGB2*, *C3*, *C1S*, *CFH*, *CD47*, *A2M*, *PLA2G7*), ECM and adhesion (including *FBLN2*, *PCDH18*, *COL1A1*, *COL5A2*, *COL6A3*, *SPP1*, *DPT*), and cell cycle (including *CCND2*, *EMP1*, *ITGB*, *RGCC*, *SETDB2*). 494 genes were consistently down-regulated in the non-treated group, which related to transcription (including *FOXA1*, *MED27*, *ZBTB45*, *ZNF593*, *ATF5*), and protein processing (including *TUBA3C*, *TUBA1C*, *UBE2L6*, *TOMM22*, *OS9*, *USP5*).

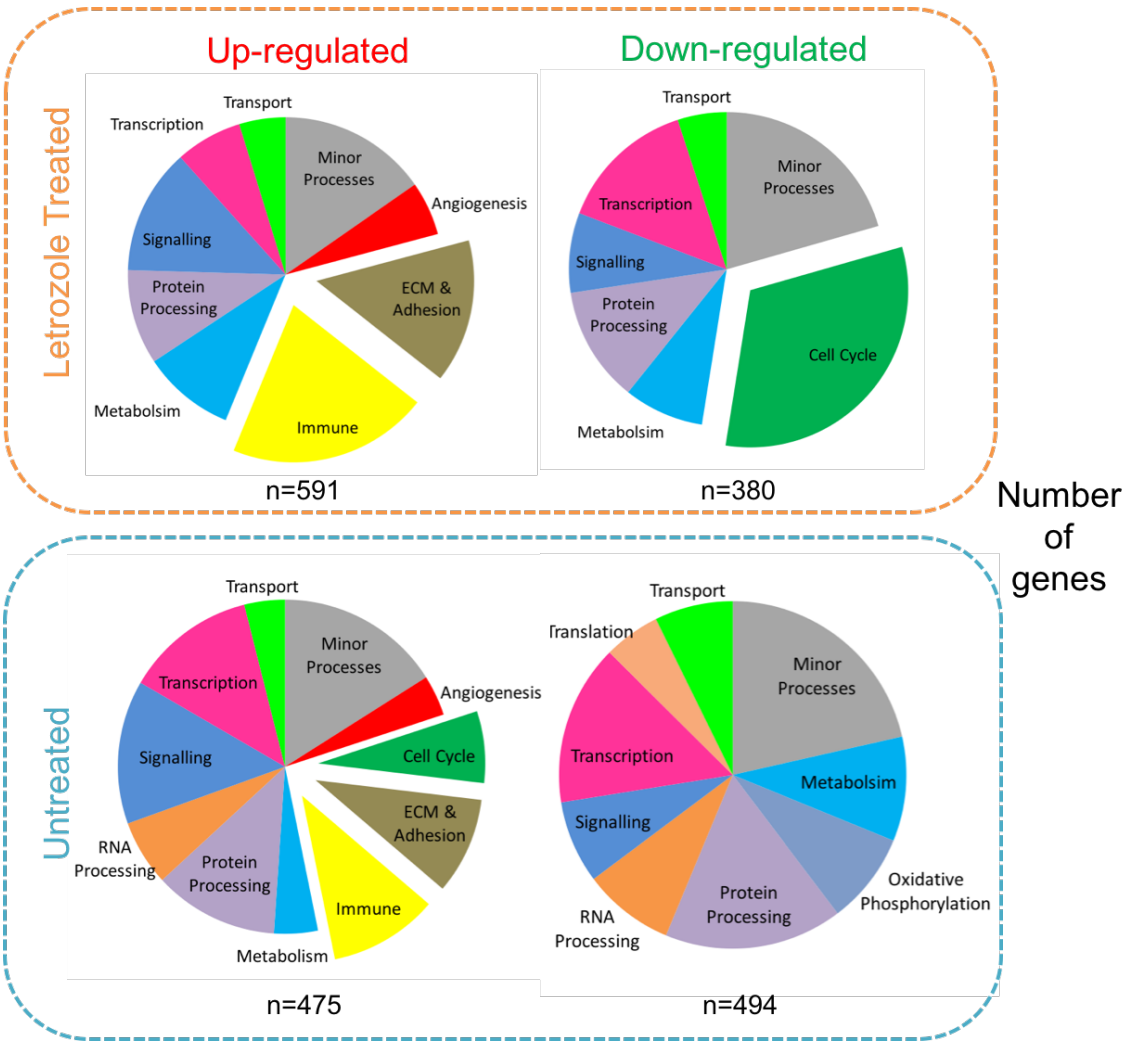

Figure 2-2. Functional analysis of most changed genes in treated and non-treated tumours

2.3.2 Common changes between treated and non-treated tumours

Comparison of genes changed in the treated versus non-treated (RP: FDR=0.05) patients, revealed overlap in only 14%, figure 2-3. In the non-treated group, 844 genes were down regulated and 784 up-regulated between the 2 biopsies. In the letrozole treated group, 633 genes were down regulated and 901 up-regulated in response to treatment. Common to both groups were 291 genes up-regulated at second biopsy, reflecting the immune, ECM and cell adhesion genes described above. 157 genes were down-regulated in both groups at second biopsy, functionally enriched for transcription and minor processes.

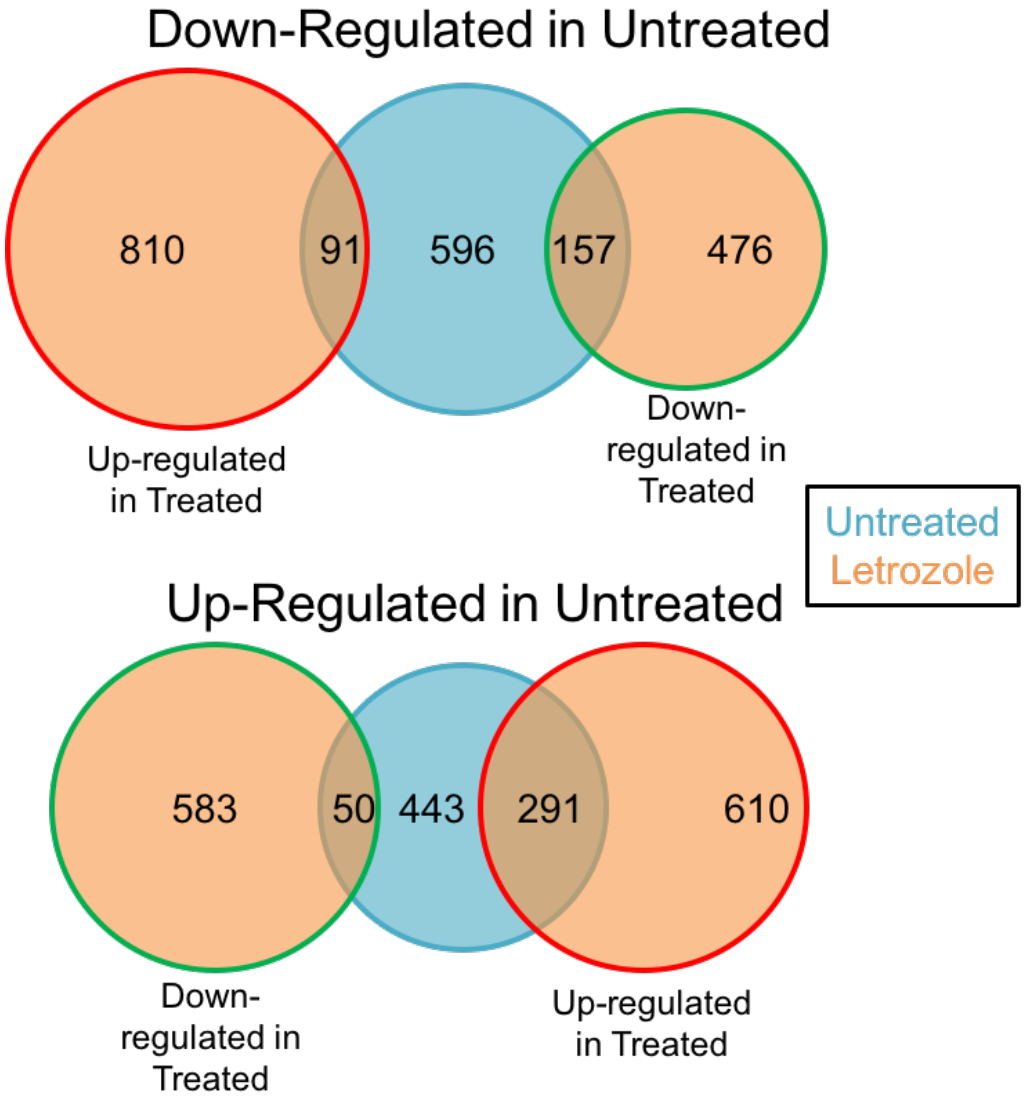

Figure 2-3. Venn diagram of common differentially-expressed genes in treated and non-treated tumours.

2.3.3 Changes in non-treated tumours

Unsupervised hierarchical clustering (HCL) of the 500 most variable genes in the non-treated samples, at core biopsy and at theatre, revealed matched samples from the same patient clustered together in 18 of 25 (72%) patients, figure 2-4. In 7 patients a biopsy more resembled a biopsy from another patient tumour, rather than itself, at a later time.

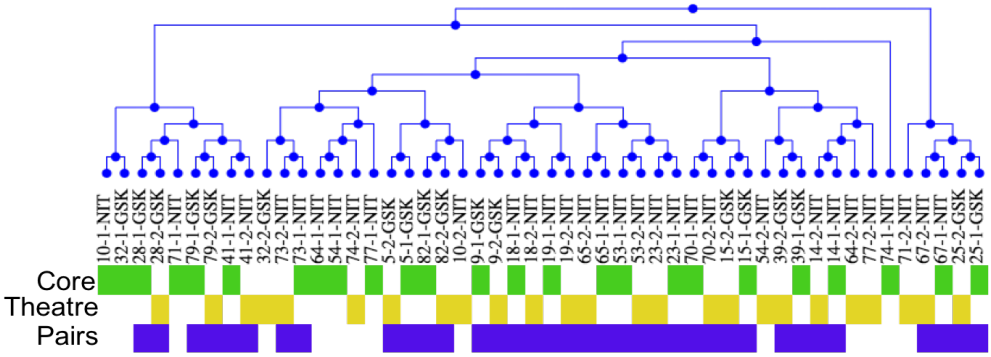

Figure 2-4. Unsupervised HCL of 500 most variable genes in non-treated group
Columns represent patients, patient IDs below dendrogram
Top row (green) - core biopsies
Middle row (gold) - theatre resection specimens
Bottom row (purple) - highlights cases where both biopsies from same patient cluster together.

Comparing the 7 patients where core and theatre biopsy did not cluster together, the 'changed' group, to those 'unchanged', revealed these patients were younger; median age 45 years (range 28-83) vs 65 (28-88), p=0.16; and were more likely to have a lobular rather than ductal cancer; 3 of 7 (42.9%) vs 3 of 18 (16.7%) p=0.5. Neither of these observations reached statistical significance however in this small sample (unpaired t test with Welch correction).

They were well matched otherwise in terms of:
tumour size (median 21mm changed vs 20.5mm unchanged);
inter-biopsy time interval (median 24 days changed vs 21.5 days unchanged);
lymph node positivity; (2 of 7 (28.6%) changed vs 5 of 18 (27.8%) unchanged);
tumour grade (5 of 7 (71.4%) grade 2 changed, 11 of 18 (61.1%) grade 2 unchanged),
ER positivity (all patients) and HER2 status (0 in changed, 6/18 (33.3%) in unchanged).

2.3.4 Effect of time on non-treated tumours

Non-treated patients were arranged in order of the length of time interval between baseline and second biopsy (days) to assess changes in genes over time, figure 2-5.

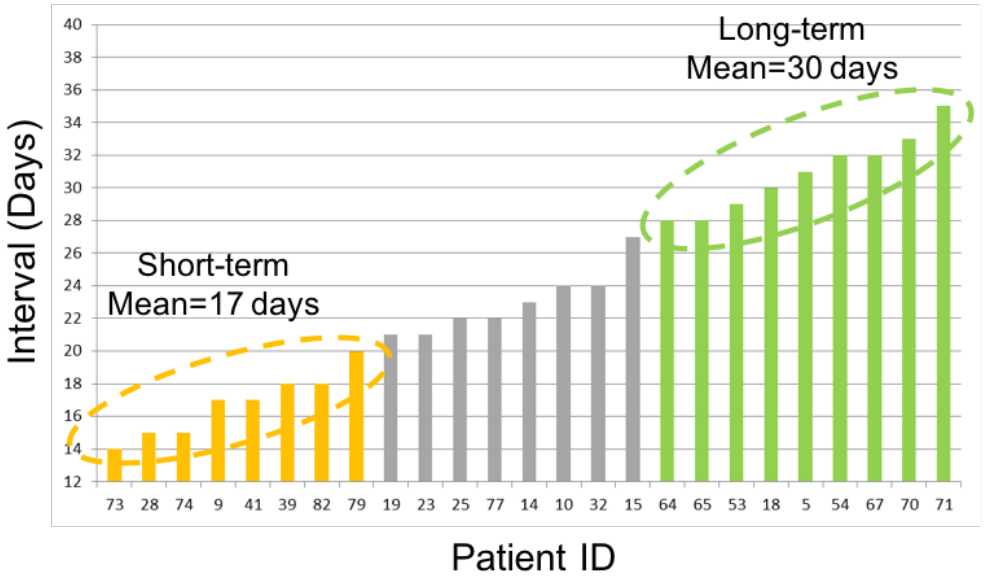

Figure 2-5. Interval between baseline and second biopsy

The 8 shortest inter-biopsy interval patients (yellow; mean 17 days) were compared with those with the longest inter-biopsy interval (green; mean 30 days), (RP: FDR 0.05).

The overlap in changed genes was very minor between short and long inter-biopsy interval groups (6.2%). In the longer interval group, significantly more changed genes were identified, both up and down-regulated, figure 2-6.

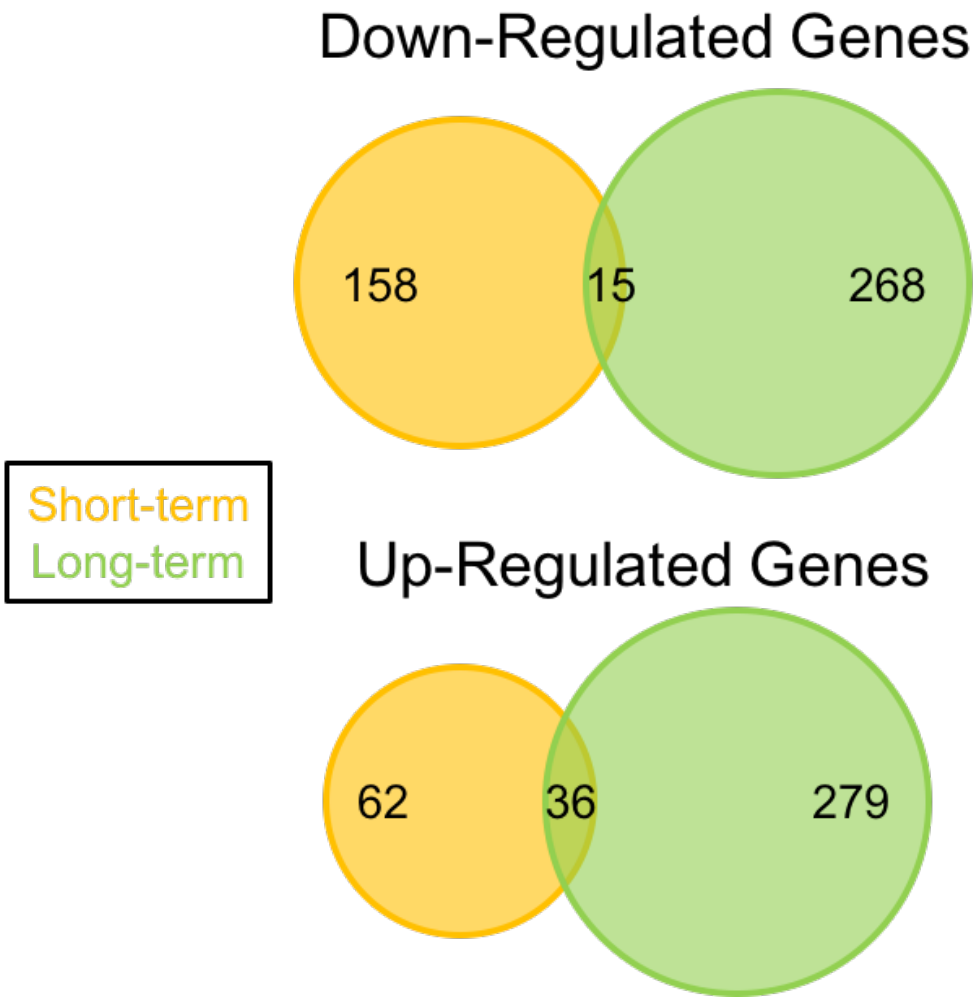

Figure 2-6. Venn diagram of common differentially-expressed genes in non-treated tumours, between short and long-term inter-biopsy time intervals

Functional analysis using PANTHER highlighted that longer inter-biopsy interval genes were enriched for transcription, immune response, intracellular signalling, ECM and adhesion, figure 2-7.

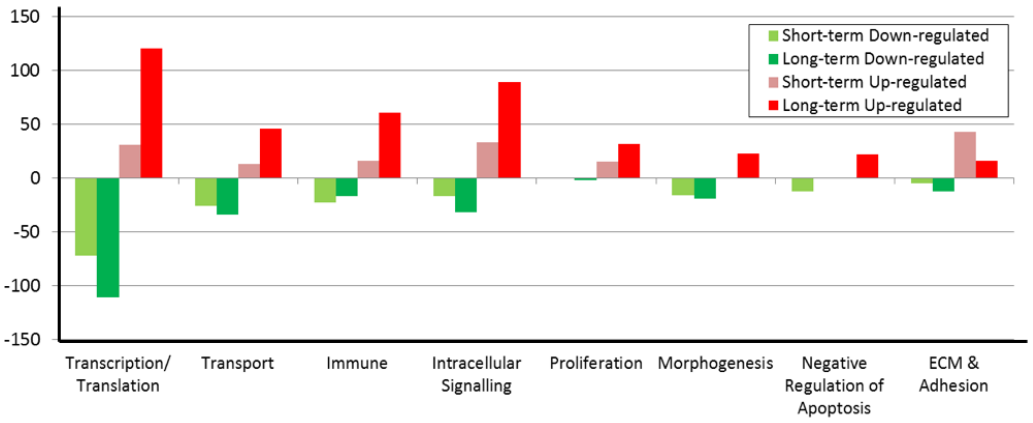

Figure 2-7. Numbers of up (red) and down-regulated (green) genes in enriched processes at short and long-term intervals in non-treated tumours.

A heatmap was generated comparing treated and non-treated patients by time interval groups. The non-treated tumours did not show significant down-regulation of proliferation genes, unlike the treated samples, unsurprisingly. Increased expression of immune and ECM associated genes was observed in the treated tumours. Similar changes were not found in non-treated tumours at the short time interval, however they were seen after the longer inter-biopsy time interval, figure 2-8.

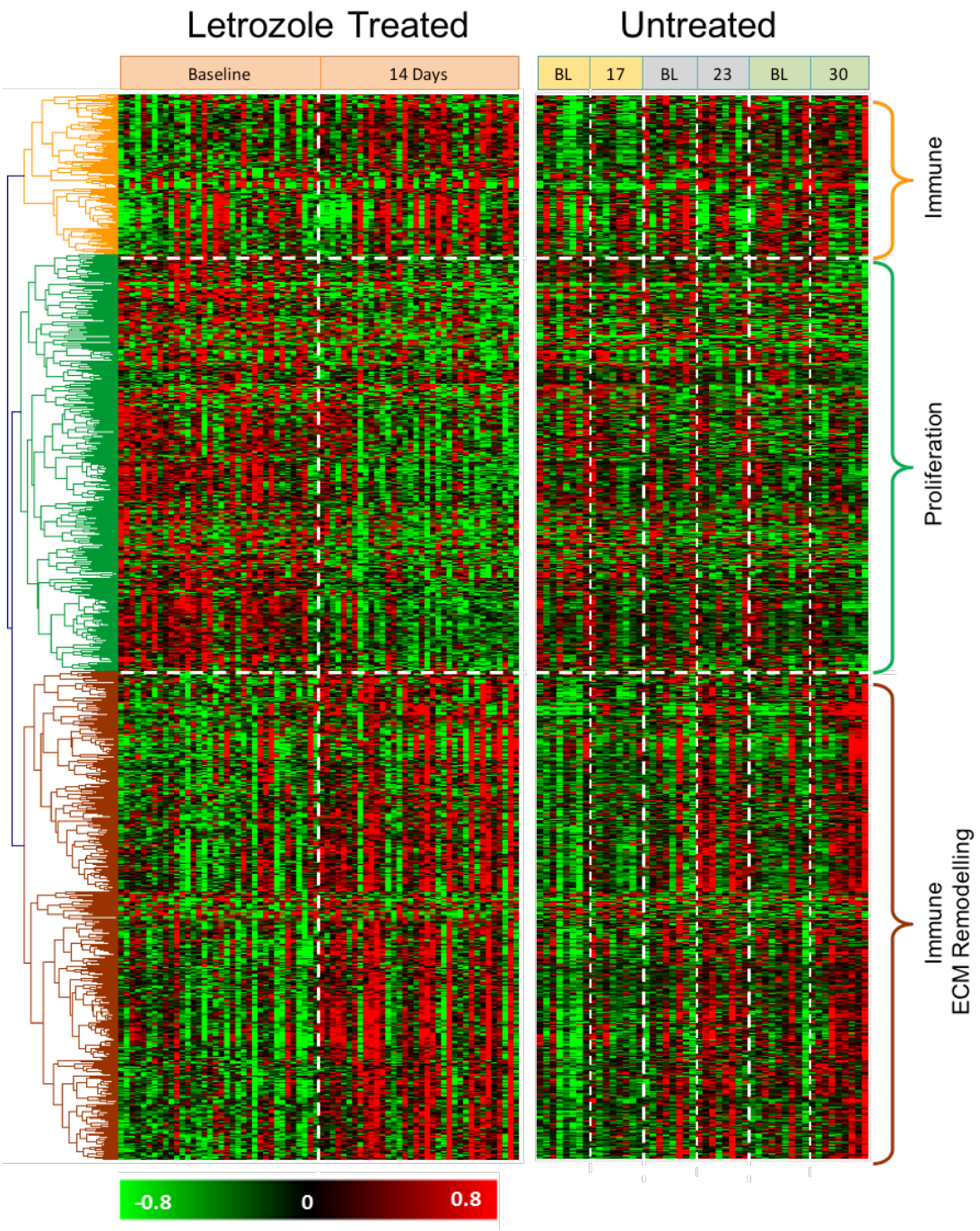

Figure 2-8. Heatmap of most differentially-expressed genes in treated compared with non-treated tumours at different inter-biopsy time intervals

Samples are ordered left to right by time interval, at baseline (BL) and mean number of days to second sample. Colours represent change in expression (after / before) with red denoting higher expression and green lower expression.

2.4 Discussion

Down-regulated gene processes, particularly proliferation related, were seen in letrozole treated tumours but not in non-treated. This is not unexpected, but adds validity to any neoadjuvant measure of response using proliferation as a surrogate biomarker. Previous studies have demonstrated increased Ki67 at second biopsy in the same tumour with no intervening treatment [247, 249]. In this cohort however, *MKI67* was not a gene which featured in those consistently changed between baseline and surgery across all non-treated samples.

In the 4-gene model predictive of response to neoadjuvant letrozole [149], the measure of 2 genes at diagnosis (*IL6ST* and *NGFRAP1*) and 2 proliferation genes (*ASPM* and *MCM4*) after 2 weeks of letrozole is predictive of response to treatment with 96% accuracy. However, in the same cohort, Ki67 assessed by IHC, which is considered a gold-standard proliferation marker, was not significantly different at 2 weeks in responding and non-responding groups. Using mRNA measures of proliferation may be a more sensitive method of tumour proliferation assessment than IHC, not least considering the problems in the reproducibility and cut off measures to assess Ki67 by IHC. We have demonstrated down-regulation of another common proliferation marker, *AURKA*, in letrozole treated tumours, which was not mirrored in the non-treated cohort. In a panel of proliferation associated genes, there was no consistent trend identified between baseline and surgery in non-treated tumours, compared with consistent down-regulation of these genes in letrozole-treated patients (see Supplementary 8-1). The non-treated cohort in this study has utility for all future neoadjuvant trials representing a significant negative control cohort as a comparison for newly proposed on-treatment biomarkers.

The overlap between non-treated and treated tumours is small; with only around 14% of genes changing over time common to both cohorts. The non-treated cohort validates the conclusion that most genes changed in letrozole-treated tumours appear to be directly related to treatment, and are not consistently changed in non-treated tumours. This confirms the value and validity of an on-treatment biopsy to assess response.

With longer time interval between biopsies the number of genes changing in non-treated tumours increases. This is similar to previous observations in letrozole treated tumours, where more genes changed after longer periods of treatment [145]. Genes up-regulated after longer non-treatment interval are related to immune processes, adhesion, and extracellular matrix.

Genes related to the same processes also increase in the comparative letrozole treated cohort at an earlier stage, and also in a previous study in response to neoadjuvant anastrozole and letrozole [146], albeit in greater numbers than in non-treated patients. Genes which are normally kept repressed by ER may become induced when ER loses its inhibition of them, either in E2-ER binding in non-treated tumours, or in response to endocrine therapy in treated tumours. This may explain the up-regulation of certain genes in both cohorts. Jeselsohn *et al* demonstrated similar upregulation of immune related genes between non-treated core and surgical specimen over a mean of 30 days, comparable to our findings [260]. This may reflect the tumour growing over time; or remodelling itself in response to an insult (biopsy related) in non-treated; or remodelling in response to aromatase inhibition in treated patients. We were unable to confirm the wound healing signature that Morrogh described in response to biopsy. The 2 genes highlighted in their study, *FOSB* and *MLL* did not feature in the most differentially expressed genes between non-treated core and surgical specimen in this cohort.

In unsupervised hierarchical clustering, biopsies from the same tumour cluster together in 18 of 25 (72%) patients. This is comparable to concordance in a similar pre-operative study where 16 of 23 (70%) and 41 of 56 (73%) matched samples from the same patient clustered together [261]. In our cohort, the changed tumours tended to be in younger patients and were more likely to be lobular than ductal. 3 of the 7 patients, in whom samples did not cluster together, were also in the longer 'inter-biopsy interval' group. This shows that in the absence of treatment there are still active molecular processes, which increase over time. Comparing progression free and overall survival in these against non-changed tumours, did not reveal any adverse prognosis. The small size of this cohort however (7 patients), and relatively short follow up period available (median 27 months, range 1-43 months) means these observations lacked significance. One could theorise this may reflect tumour evolution to a more aggressive subtype. We have demonstrated discordance in molecular subtype, in core biopsy to surgical specimen with no intervening treatment, in 7 of 37 patients (18.9%) [262]. The subtype changes in these patients however were not consistent, so we are unable to draw significant conclusions. This would be worthy of further assessment in a larger cohort however, as it could significantly alter adjuvant treatment decisions.

A further limitation of this study is comparing 2 different methods of biopsy. In the letrozole-treated cohort, the second biopsy was a further core biopsy, whereas in the non-treated cohort it was an excised surgical specimen. This would inevitably cause slight differences in the way the specimen was processed after acquisition; eg likely longer time to freezing, but we are

unable to quantify or confirm this effect. Previous work has demonstrated high concordance in gene expression profiles between core and surgical biopsies [263] however. Additionally, the numbers of samples at each time point are different in treated and non-treated; a further potential bias. Our cohort was reasonably well matched in clinicopathological features, however we did observe significantly more T4 tumours in letrozole treated, and more T1 tumours in non-treated patients, which will of course be reflected in gene expression profiles.

2.5 Conclusions

We have demonstrated even in the absence of treatment breast cancers are evolving and remodelling; an effect which increases over time. There is increasing evidence of the benefit of short course perioperative AI treatment for a matter of weeks, whilst patients undergo essential staging investigations and planning for surgery. On-treatment biopsy is more reflective of long term outcome than baseline, therefore samples collected at baseline and again at surgery provide crucial prognostic and predictive biological endpoints. Further work is needed in a larger cohort to assess molecular changes in the surgical specimen, which may alter adjuvant treatments. However, this study represents one of the largest cohorts to date assessing gene expression profiling with no treatment, and validates the use of an on-treatment biopsy as true measure of drug effect, or assessment of prognosis.

3 Changes in *PIK3CA* mutation status are not associated with recurrence, metastatic disease or progression in endocrine treated breast cancer

3.1 Introduction

The phosphatidylinositol 3-kinase (PI3K) / AKT / mTOR pathway plays an important role in proliferation, migration and survival in breast cancer. Oncogenic activation of the pathway can occur through increased kinase activity due to missense mutations of the phosphatidylinositol (4,5)-bisphosphate 3-kinase, catalytic subunit α (*PIK3CA*) gene [264]. Mutations in *PIK3CA* therefore play a significant role in carcinogenesis and progression of breast cancer, and previous studies indicate that mutation occurs in 8-40% of breast tumours [67, 265-271]. *PIK3CA* mutations are more common in hormone receptor-positive breast cancers than in HER2-positive or basal-like and triple negative tumours [67, 272].

PI3K pathway activation is implicated frequently in breast cancer [273], and *in vitro* studies have demonstrated pathway activation is associated with endocrine resistance and oestrogen independent activation of ERα [274, 275], figure 3-1. In the neoadjuvant setting, however Ellis *et al.* reported only a modest negative association between *PIK3CA* mutation and clinical response to aromatase inhibition [276]. Targeting the pathway with inhibitors of PI3K and mTOR combined with anti-oestrogen therapy does improve endocrine responsiveness in breast cancer cell lines [275]. *In vivo,* drugs such as everolimus, targeting the mTORC1 component of this mTOR pathway, provide an enhanced anti-tumour response when combined with aromatase inhibition in the neoadjuvant [217] and metastatic setting [218].

The clinical and pathological relevance of *PIK3CA* mutations is poorly understood. Associations between *PIK3CA* mutation and large tumour size, higher grade, nodal involvement and poorer survival have been described [277], however it is far from clear whether there is any significant correlation with clinical outcome [278]. One study in fact shows *PIK3CA* mutation to be associated with favourable clinicopathological disease features and improved survival [266].

The phosphatase and tensin homolog deleted on chromosome ten (PTEN) protein is a potent suppressor of PI3K pathway activity. Loss of functional PTEN leads to stimulated cell growth

and survival [279]. Reduced PTEN protein expression has also been implicated in endocrine resistant breast cancer and is associated with shorter disease free survival [280].

The relationship between *PIK3CA* mutation and PTEN is poorly understood with some descriptions of *PIK3CA* mutations and PTEN loss occurring only independently [267], and others where they coexist [281]. A significant proportion of breast cancers do evade anti-oestrogen therapy through dysregulation of PIP3 either through mutated *PIK3CA* or loss of functional PTEN [282, 283].

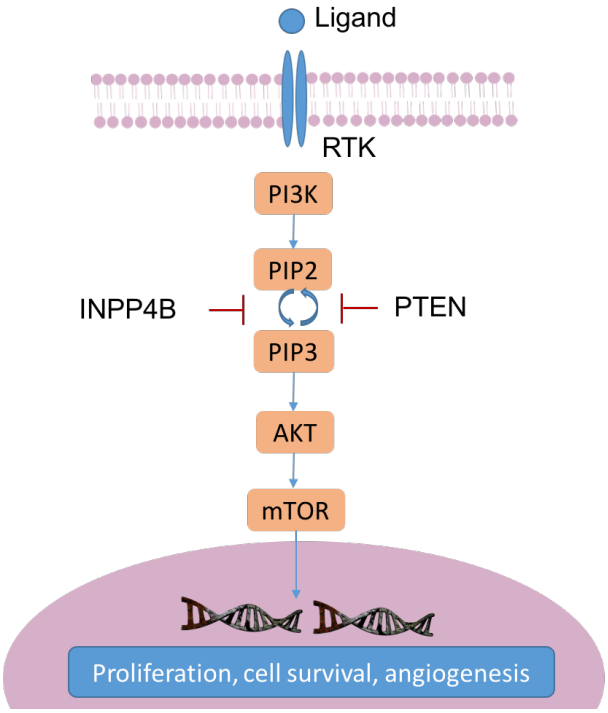

Figure 3-1. Schematic diagram of PI3K pathway.
PI3K is activated by ligand binding to RTK or mutation of PIK3CA or PIK3CB. PI3K phosphorylates PIP2 to PIP3, which recruits AKT, which in turn activates mTOR. Inhibition of the pathway is maintained by lipid phosphatases PTEN and INPP4B which dephosphorylate PIP3 and PIP2 respectively.
RTK – Receptor Tyrosine Kinase, PI3K – phosphatidylinositol 3-kinase,
PIP2 – phosphatidylinositol 4,5-bisphosphate,
PIP3 - phosphatidylinositol 3,4,5-trisphosphate,
mTOR – mammalian target of rapamycin,
INPP4B – inositol polyphosphate-4-phosphatase,
PTEN – phosphate and tensin homolog

Endocrine resistance is problematic in the management of ER+ breast cancers. Loss of ERα [284] or mutations in *ESR1* [221, 285] in metastatic tumours, leading to ligand independent activation of ER, are possible reasons for endocrine resistance. Discordance in protein receptor status (ER, PgR and HER2) between primary and metastatic breast tumours has been widely reported [286], and alternative receptor status in recurrent disease could lead to a different treatment plan for as many as one in six patients [287]. Recent recommendations state metastases should be biopsied in order to best personalise treatment in these patients, though this is not yet part of routine clinical practice [288].

As *PIK3CA* mutation and PTEN loss are being investigated as potential predictors of response to novel PI3K inhibitors, and as possible mechanisms of endocrine resistance, it is prudent to know whether *PIK3CA*/PTEN status in primary breast tumours is concordant with that in metastatic lesions. Gonzalez-Angulo *et al*. found similar rates overall of *PIK3CA* mutation in primary tumours and distant metastases (40.4% and 42% respectively) and PTEN loss (30.4% and 25%) in their study of 50 matched patient samples of primary and asynchronous distant breast cancer recurrences. However within individual patients there was discordance between primary and recurrent *PIK3CA* mutation status in 18%, and PTEN status in 26% [289]. Dupont Jensen *et al.* found *PIK3CA* mutations in 45% of primary tumours and only 34% of synchronous metastatic axillary nodes. In later metastases they found a higher incidence of *PIK3CA* mutations, postulating mutations may be acquired in disease progression. Indeed they even found heterogeneity within areas of the same primary tumour in 4 of 10 patients using laser capture microdissection [290]. PTEN was also significantly higher in metastases than in the primary tumour.

The aim of this study was to assess *PIK3CA* mutations and PTEN status in a unique cohort of patients with ER+ breast cancer who have developed resistance to endocrine therapy. This study utilises an exclusive cohort of matched paired samples from patients with ER+ breast cancer at both diagnosis and on progression, at the time of recurrence or development of a new breast cancer following endocrine treatment, in order to assess whether mutation status changes over time or is constant. This may provide further verification for the role of novel PI3K inhibitors as adjunctive treatment of endocrine resistant disease.

3.2 Methods

3.2.1 Patient samples and endocrine treatment

Paired formalin fixed paraffin embedded (FFPE) tissue samples were located for a series of 120 patients treated at the Western General Hospital, Edinburgh between 1990 and 2011 for a primary and recurrent breast cancer. All patients had hormone receptor positive disease at first diagnosis and received endocrine therapy; either tamoxifen, letrozole, anastrozole, exemestane or a combination of these as adjuvant or primary endocrine therapy, then developed disease progression, recurrence or a further primary breast cancer. Samples were taken at diagnosis and from five classes of secondary event;

local recurrences (in breast tumour recurrence, or chest wall recurrence);

metastatic recurrences (axillary or supraclavicular fossae nodal metastases);

local progression (patients treated with primary endocrine therapy who initially responded well and then progressed); and

from second breast cancers (ipsilateral second primary breast cancers, or contralateral new primaries).

Where the secondary event was a recurrent tumour in the treated breast, pathology and mammography records were assessed to determine whether these second tumours represented in breast local recurrences or true second primary breast cancers. Clinicopathological disease features, tumour characteristics, treatment regimens and survival data was traced from electronic and paper case note record review. Pathology reports issued at the time of treatment were prepared by dedicated breast pathologists. Data was incomplete across every field in 17 of the original 120 samples, mostly due to historic case records being unavailable. All patients gave informed consent to be included in the study which had been approved by the local ethics committee (LREC; 2001/8/80 and 2001/8/81).

3.2.2 *PIK3CA* mutation status

Five 5-µm sections were cut from each FFPE tumour block and DNA extracted using the QIAamp FFPE kit (Qiagen) after deparaffinisation with Envirene, as described previously [291]. 150ng of DNA from each sample was profiled using the Roche *PIK3CA* Mutation Test, a multiplex PCR assay designed to detect 8 mutations in exons 7, 9 and 20 in the *PIK3CA* gene [292]. The analytical sensitivity of the assay, defined as ≥95% mutation detection rate, has previously been shown to be ~5% mutant allele in breast cancer FFPE tumour sections representing the 3 most prevalent *PIK3CA* mutations (H1047R, E545K, and E542K).

3.2.3 Immunohistochemistry

Oestrogen receptor status was determined as part of routine clinical evaluation using the Allred classification system. HER2 status assessed by IHC and FISH was determined routinely on all patients diagnosed after 2004 when this entered routine clinical practice. PTEN IHC was conducted at a central laboratory (Histogenix, Belgium) on FFPE tumour samples using the Discovery XT automated staining platform (Ventana, Tucson, AZ). Antigen retrieval was performed with Cell Conditioning I reagent (CC1, Ventana). The primary antibody was from Cell Signaling Technologies (clone 138G6). Reactions were developed using the UltraMap DAB detection system (Ventana), and counterstained with Hematoxylin II (Ventana). PTEN was scored in a semi-quantitative fashion using a H-score method to account for heterogeneity of expression, as described previously [291], and staining of normal stromal components of each sample was used as an internal control for sample integrity. In subsequent statistical analyses, a H-score of 100 or less was used as the cut-off to designate loss of PTEN.

3.2.4 Survival Analysis

Progression free survival was calculated from the date of biopsy confirming recurrent disease, compared to the date of the original sample. Overall survival was calculated from date of death in the patient medical records compared with date of primary sample. Survival data was available in 88 patients with known *PIK3CA* status at primary event and 44 patients with both *PIK3CA* and PTEN status known at primary event. Progression free and overall survival was assessed in mutant and non-mutant groups using Kaplan-Meier survival analysis with log rank (Mantel Cox) comparison of curves. Features of patients and tumours at diagnosis by mutation group were compared using analysis of variance (ANOVA) for age and tumour size, and for all other features using Fisher's exact test following construction of contingency tables. Comparison of recurrence type by mutation status was performed with Fisher's exact test in contingency tables. All statistics were performed in GraphPad Prism version 6.00 for Windows, (GraphPad Software, La Jolla California USA).

3.3 Results

PIK3CA mutation status and/or PTEN immunohistochemistry was obtained for 96 of the original 120 patient cohort. Of these 89 had *PIK3CA* mutation status available from both the primary and secondary events; and 42 had PTEN immunohistochemistry from both primary and secondary events. One patient had only DCIS in the biopsy at diagnosis and a contralateral invasive cancer as secondary event, and was excluded from further analysis. All other patients had histologically confirmed invasive breast cancer which was hormone receptor positive at diagnosis. Patient clinicopathological disease features and treatment regimens were recorded from case note review for the available 96 cohort and tabulated by mutation status, table 3-1. The mean age of the cohort was 66.3 years at diagnosis; 78% of tumours were ductal histology; 79% nuclear grade 2-3 and 85% had high ER expression (Allred scores 6-8). Twenty-eight patients (29.2%) received endocrine therapy as primary or neoadjuvant endocrine treatment, whilst all others received it as adjuvant therapy following surgery and tumour resection.

Twenty-seven of the adjuvant therapy group (39.7%) received additional systemic chemotherapy with an anthracycline based +/- taxane regime. Fifty-one (75%) received regional radiotherapy following surgery for their primary cancer. The interval between primary and secondary event ranged from 69 days to 18.5 years with a median of 2.4 years.
Within mutation groups there were no significant differences in tumour size, grade, histology, lymphovascular invasion, nodal status or ER Allred score. There was no correlation between ER Allred score and PTEN expression or *PIK3CA* mutation. PTEN loss however was found in a significantly older subgroup of women compared with those with *PIK3CA* mutations (mean 77.3 vs 62.1 years, one-way ANOVA with Tukey multiple comparisons, $p=0.05$) although in this study our PTEN loss cohort is of limited size (7 women).

	Total		PIK3CA mutations			PTEN low/loss		
Variable	n	(%)	n	(%)		n	(%)	
	96	(100)	38/89	(42.7)		7/42	(16.7)	
Age, years								
Mean	66.3		66.4			77.3		**p=0.05**
SD	15.6		15.7			9.8		
Median	68		68			80		
Range	25-94		25-94			57-86		
Histology								
Ductal	75	(78.1)	26	(68.4)	p=0.27	7	(100)	p=0.34
Lobular	6	(6.25)	4	(10.5)	p=0.47	0		
Mixed	2	(2.1)	1	(2.6)		0		
Tubular	4	(4.2)	3	(7.9)		0		
Other	1	(1)	1	(2.6)		0		
Not recorded	7	(7.3)	3	(7.9)		0		
DCIS	1	(1)	0			0		
Tumour grade								
1	12	(12.5)	6	(15.8)	p=0.2	0		
2	33	(34.4)	14	(36.8)	p=0.66	3	(42.9)	p=0.35
3	34	(35.4)	12	(31.6)	p=1	4	(57.1)	p=1
Not recorded	17	(17.7)	6	(15.8)		0		
Tumour size (mm)								
Mean	30.3		29.3			39.9		p=0.38
SD	18.4		17.8			23		
Median	24.5		23			30		
Range	5-105		5-70			12-80		
Lymphovascular invasion								
Positive	22	(22.9)	8	(21.1)		1	(14.3)	
Negative	61	(63.5)	25	(65.8)	p=1	6	(85.7)	p=0.67
Not recorded	13	(13.5)	5	(13.2)		0		
Nodal Staging								
Node negative	45	(46.9)	14	(36.8)		2	(28.6)	
Node positive	36	(37.5)	18	(47.4)	p=0.3	4	(57.1)	p=0.4
N1	28	(29.2)	14	(36.8)		3	(42.9)	
N2	6	(6.3)	2	(5.3)		1	(14.3)	
N3	2	(2.1)	2	(5.3)		0		
Nx	15	(15.6)	6	(15.8)		1	(14.3)	
ER Allred Score								
Low (1-5)	14	(14.6)	3	(7.9)		1	(14.3)	
High (6-8)	82	(85.4)	35	(92.1)	p=0.4	6	(85.7)	p=1
HER2								
Positive	13	(13.5)	2	(5.3)		2	(28.6)	
Negative	50	(52.1)	26	(68.4)	**p=0.03**	5	(71.4)	p=0.62
Unknown	33	(34.4)	10	(26.3)		0		
Endocrine treatment								
Anastrozole	10	(10.4)	3	(7.9)	p=0.78	0		
Letrozole	37	(38.5)	12	(31.2)	p=0.55	6	(85.7)	**p=0.04**
Tamoxifen	37	(38.5)	20	(52.6)	p=0.17	1	(14.3)	p=0.24
Combination of tamoxifen & AI	5	(5.2)	1	(2.6)		0		
Multiple AIs	2	(2.1)	0			0		
Unknown	5	(5.2)	2	(5.3)		0		

Table 3-1. Patient and tumour characteristics at primary event

DCIS – ductal carcinoma in situ, AI – aromatase inhibitor

3.3.1 *PIK3CA* mutation status

PIK3CA mutation status was determined for both primary and matched interval samples for 89 patients. Of these, 38 (42.7%) had at least one *PIK3CA* mutation at diagnosis, and 37 (41.2%) had a mutation present in tissue taken at the time of recurrence. The most common mutations in order were H1047R (35 samples), E545K (17 samples), H1047L (11 samples), E542K (5 samples), E545A (2 samples), C420R (2 samples). A further 3 cancers had a combination of mutations. The incidence of each mutation was similar at both primary and secondary event, figure 3-2.

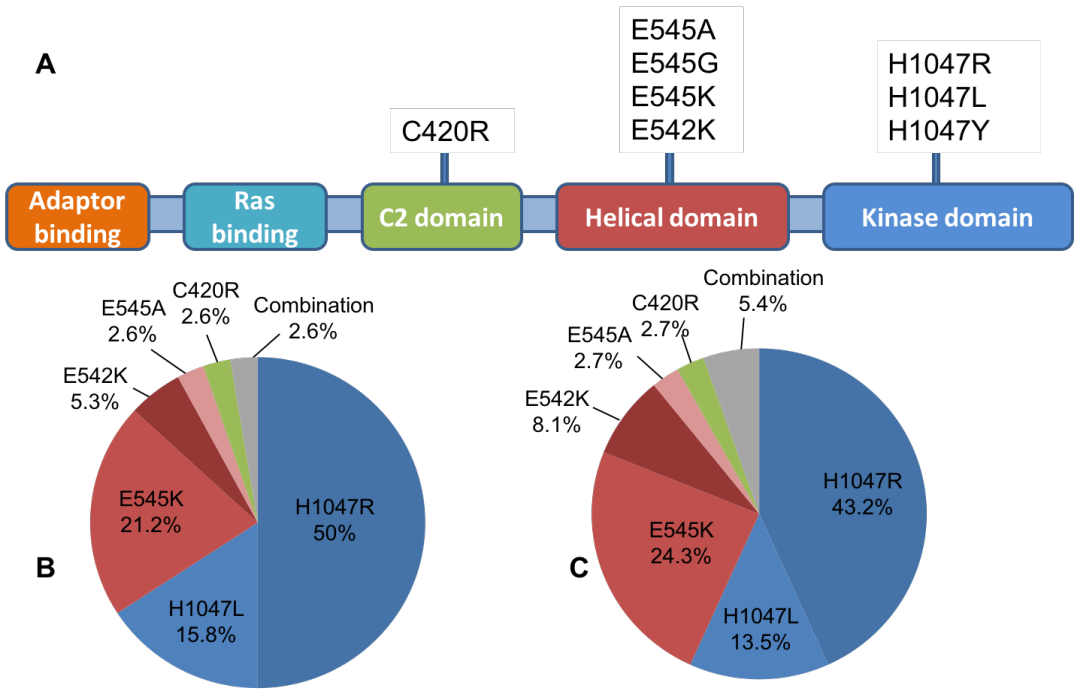

Figure 3-2. PIK3CA mutation status in primary and secondary breast tumours treated with endocrine therapy

***A**: PIK3CA protein and functional domains demonstrating position of mutations assayed in this study.*

***B**: Frequency of mutations in this cohort in primary and **C**: secondary tumours.*

Across the cohort only 14 patients (15.7%) had a change in their *PIK3CA* mutation status between primary and secondary event. The type of recurrence in those patients with altered *PIK3CA* status is demonstrated in Table 3-2. A change in *PIK3CA* status was demonstrated in more patients who developed a second primary breast cancer in either the treated (3/5, 60%; Fisher's exact test p=0.03) or contralateral breast (5/6, 83.3%; Fisher's exact test p=0.0003); than in any other type of secondary event.

Secondary Event	n	PIK3CA gain	PIK3CA loss	Change in PIK3CA mutation status (%)	P value
Progression on primary endocrine therapy	28	0	1	3.6	**p=0.03**
Metastatic nodal recurrence	17	1	1	11.8	p=1
Local recurrence	32	3	0	9.4	p=0.24
Contralateral new primary breast cancer	6	2	3	83.3	**p=0.0003**
Ipsilateral 2nd primary breast cancer	5	2	1	60	**p=0.03**

Table 3-2. PIK3CA mutation gain or loss between primary and secondary event

3.3.2 PTEN activity

PTEN status was available at both primary and secondary event in 42 matched patient samples. PTEN loss, defined as H score 100 or less, was similar at both time-points, seen in 7 (16.7%) patients at primary event and 8 (19%) at secondary event. Across individual patients there was little variation in PTEN status, with only 4 patients who had PTEN wild type tumours at primary event acquiring loss of PTEN by secondary event, using the H score 100 cut-off. When looking at absolute values however variance between primary and secondary tumour in the majority of patients who changed from WT to PTEN loss and vice versa was small (difference in H score <100). Two patients were exceptions to this with large changes in PTEN expression (100 at primary event to 290 at secondary event; 300 at primary event to null at secondary event), figure 3-3.

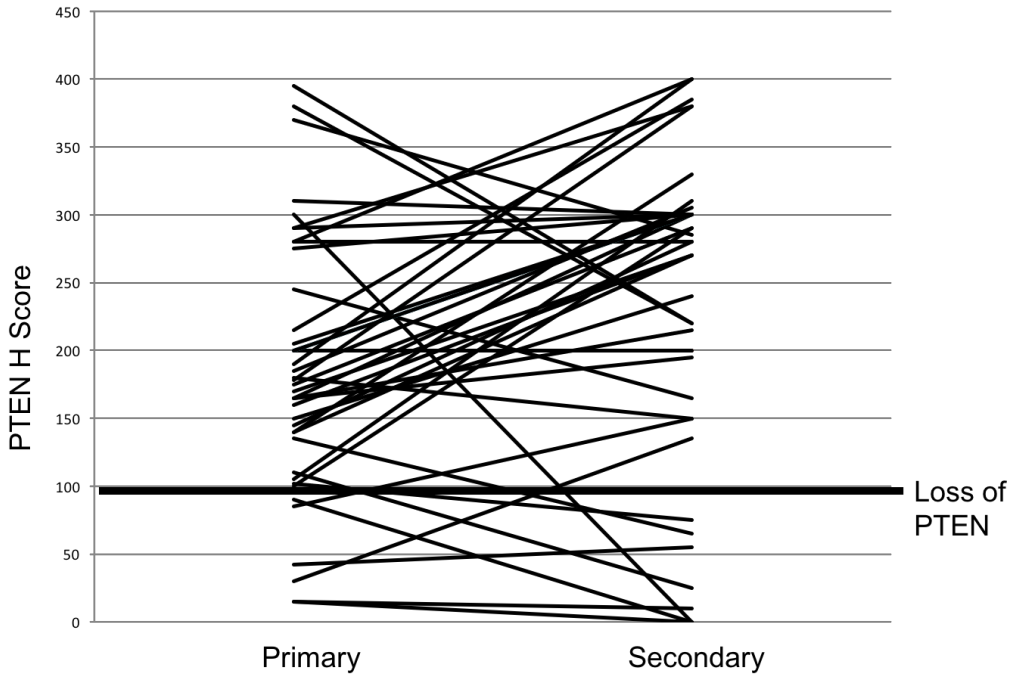

Figure 3-3. PTEN expression by H score at primary and secondary event
Changes in PTEN expression between primary and secondary breast cancer following endocrine treatment. Line plot shows matched primary and secondary breast tumours from patients treated with endocrine therapy. Immunohistochemistry was quantified by H-score

Assessing PTEN as a continuous variable demonstrated a slightly higher PTEN value in metastatic samples (mean H score 227.3, SD117.2); compared to primary samples (mean 185, SD 95, unpaired t test with Welch's correction p=0.07). The number of PTEN null cases increased at secondary event compared to primary (3 of 42 at recurrence, (7.1%); 0 at primary event). There were also more cases of over expression (>200) of PTEN at secondary event (27 of 42, 64.3%) compared to primary event (17 of 42, 40.5%). Many of the tumours with PTEN loss were located in women whose disease progressed whilst on primary (neoadjuvant) endocrine therapy (5 of 15 at primary event, 6 of 15 at secondary event), table 3-3.

Secondary Event	n	PTEN loss at primary event	PTEN loss at secondary event	Proportion of patients with PTEN loss (%)	P value
Progression on primary endocrine therapy	15	5	1	40	p=0.27
Metastatic nodal recurrence	8	1	1	25	p=1
Local recurrence	17	1	1	11.8	p=0.09
Contralateral new primary breast cancer	1	0	1	100	p=0.27
Ipsilateral 2nd primary breast cancer	0	0	0		

Table 3-3. Patients with PTEN loss compared to type of secondary event

3.3.3 *PIK3CA* and PTEN status and progression free and overall survival

The mean time to progression across the whole cohort was 3.8 years (range 69 days – 18.5 years, median 2.4 years). Patients with PTEN loss at diagnosis had a significantly shorter time to progression than those with retained PTEN (mean 1.3 years vs 4.9 years; log-rank (Mantel Cox) test p=0.006), figure 3-4A. Patients with *PIK3CA* mutations at diagnosis had a mean time to progression of 3.8 years, which was not significantly different from *PIK3CA* wild type patients (mean 4.2 years), figure 3-4B. Further analysis of patients who had results for both *PIK3CA* and PTEN status available at primary sampling (n=44) upheld this significantly shorter time to progression seen with PTEN loss, which is independent of *PIK3CA* status, figure 3-4C; although our sample size is relatively small with only 7 of 44 patients (15.9%) demonstrating loss of PTEN at primary event. There were no significant differences in overall survival between mutant and wild type groups based on mutation status at primary event in either *PIK3CA* or PTEN. There was no significant difference in progression free or overall survival in *PIK3CA* mutant tumours by location of *PIK3CA* mutation at diagnosis in this cohort (exon 9 or exon 20).

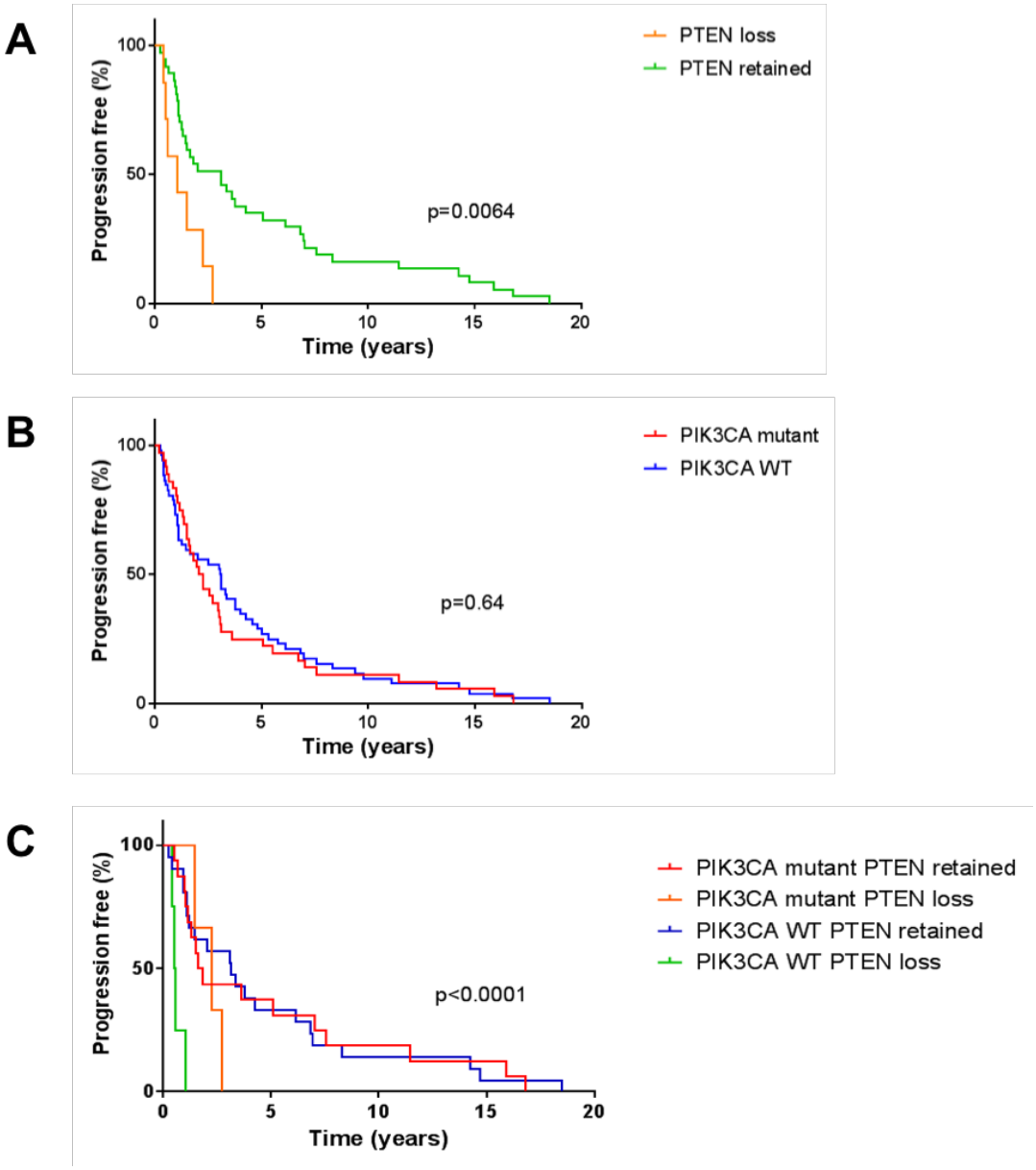

Figure 3-4. Progression free survival by PIK3CA and PTEN status at primary event.

***A**: PTEN status in primary tumour and time to recurrence (n=44).*

***B**: PIK3CA status in primary tumour and time to recurrence (n=88).*

***C**: Assessment of both PTEN and PIK3CA status in primary tumour and time to recurrence (n=44); demonstrating PTEN loss is associated with significantly shorter time to recurrence independent of PIK3CA status.*

Loss of PTEN, but not PIK3CA is associated with poor prognosis. *WT – wild type.*

3.4 Discussion

This study demonstrates that *PIK3CA* mutation and PTEN status are usually maintained in asynchronous breast cancer metastases. As the samples profiled in this study were taken from breast tumours that developed endocrine resistance during or after endocrine treatment, this does not support the hypothesis that acquirement of *PIK3CA* mutation is associated with development of endocrine resistance. In a recent study, Barbareschi *et al* found that *PIK3CA* mutations and / or PTEN loss in HER2+ breast carcinomas treated with trastuzumab are not related to resistance to anti-HER2 therapy [293]. Approximately 80% of *PIK3CA* mutations have been localised to three hot spots resulting in single amino acid substitutions; E545K and E542K in the helical domain (exon 9), and H1047R in the kinase domain (exon 20). Based upon a meta-analysis of *PIK3CA* mutations in several cancer types, Barbi *et al.* suggested that the ratio between mutation prevalence in exons 9 and 20 might be considered a signature of cancer type, although it was less clear whether an exon bias exists in breast cancer as many studies are apparently contradictory [294]. A difference between exon preference between lobular and ductal histotypes has previously been suggested [295], with mutations in exon 20 more common in ductal carcinomas and mutations in exon 9 more common in lobular breast cancers. Most previous studies have focused on just these mutational hotspots regions of *PIK3CA* in exons 9 and 20 [295, 296], however additional mutations have also been identified in exons 1, 4, 7, 13 and 18 [267]. These are rarely identified and less is known about their functional relevance. In this study 61.3% (46 of 75) mutations were observed in exon 20 (kinase domain) and 32% (24 of 75) in exon 9 (helical domain).

Our findings are consistent with a recent study which found that *PIK3CA* mutation status is of limited prognostic relevance in ER+ breast cancer patients treated with hormone therapy [278]. Our findings are also consistent with a study showing that PI3K and *AKT1* mutations occur early in breast carcinoma [265] and that these are constant over time in the majority of patients. When looking at type of metastases we have shown that *PIK3CA* mutation status is more likely to change in development of a second primary breast cancer in the treated or contralateral breast rather than in local or metastatic nodal recurrence. Tumours with *PIK3CA* mutation were most often associated with persistent ER expression. In vitro studies have shown that oestrogen deprivation increases the apoptotic effects of PI3K and dual PI3K/mTOR inhibitors in ER+ disease, providing a rationale for PI3K/aromatase inhibitor combinations as a first-line therapy [297]. Targeting *PIK3CA* mutant tumours with a PI3K pathway inhibitor and fulvestrant is therefore a feasible strategy for aromatase-inhibitor-resistant ER+ relapsed breast cancer [297].

Although our sample size was relatively small, we have described a significant association between loss of PTEN and shorter time to progression in ER+ breast cancers, similar to that observed by Shoman *et al.* [280]. It is also interesting that tumours with PTEN loss were more often seen in older women. We have demonstrated that *PIK3CA* mutation can occur in combination with PTEN loss in a small number of patients and that poorer progression free survival seen with PTEN loss tumours is maintained irrespective of *PIK3CA* status.

To measure the accurate functional activity of the PI3K pathway it may be necessary to look beyond *PIK3CA* mutations and PTEN status to aberrations of other genes including *PIK3CB*, *AKT1/2*, *PDK1* and *INPP4B*. A previously reported PI3K/mTOR-pathway gene signature (PIK3CA-GS) [298] has been used to estimate the level of PI3K pathway activation in two clinical trials of newly diagnosed ER+ breast cancer patients (n=81); one of which was randomized between letrozole and placebo vs letrozole and everolimus [299]. In the randomised dataset, the *PIK3CA*-GS could identify those patients with the largest relative decreases in Ki67 to the combination of letrozole and everolimus compared with letrozole alone. *PIK3CA* genotype was not significantly associated with any endpoint in either dataset in this study [299].

3.5 Conclusion

The acquisition of mutations in *PIK3CA* is not responsible for the development of endocrine resistance. *PIK3CA* mutation status does not change in the majority of patients who develop recurrent or progressive breast cancer. Therefore, if *PIK3CA* mutation status is predictive of response to PI3K inhibitors, then mutation status at diagnosis can be used to determine whether a PI3K inhibitor might be suitable for treatment for subsequent metastatic disease. Patients who develop new breast cancers frequently develop cancers with a different *PIK3CA* mutation status indicating that *PIK3CA* mutation status of different cancers can vary in individual patients. PTEN loss can exist in combination with *PIK3CA* mutation and is associated with significantly shorter time to recurrence in ER-positive breast cancers. These are the most comprehensive data currently available correlating *PIK3CA* status, location of disease recurrence and endocrine resistance.

4 Molecular changes in lobular breast cancers in response to endocrine therapy

4.1 Introduction

Invasive lobular carcinoma (ILC) accounts for approximately 10-15% of newly diagnosed breast cancers [300-302], affecting roughly 30,000 women annually in the United States [302] and roughly 7,000 annually in the United Kingdom [303]. ILC is classically characterised by small, regular uniform neoplastic cells that invade the stroma in a single-file pattern with cells encircling normal breast tissue [300, 301, 304, 305]. Inactivation of e-cadherin (*CDH1*) by a variety of molecular mechanisms is considered a characteristic of ILC [13, 306]. The pattern of infiltration explains why ILC is often large and why the extent of the tumour may be substantially underestimated by both physical examination and mammography [307]. When compared with the more common invasive breast cancers of no special type, also known as invasive ductal carcinomas (IDC), ILC is more likely to be oestrogen receptor-positive and of lower nuclear grade [308, 309]. Because ILC is often large at diagnosis, there are numerous reports of the response of these cancers to primary (neoadjuvant) chemotherapy [308, 309]. Patients with ILC are significantly less likely to have a pathological complete response than IDC patients. However a recent study concluded that ILC represents a heterogeneous group of tumours and the difference in response to neoadjuvant chemotherapy is largely explained by differences in molecular characteristics, particularly ER, PR and HER2, and *independent* of lobular histology [310]. The lack of understanding of lobular breast cancer is compounded by the paucity of research models (reviewed in [302]). A recent study suggested that E2 and anti-oestrogens differentially regulate ERα-mediated gene expression in ILC versus IDC cell lines [311].

Previous microarray studies of ILC tumour samples have focused on transcriptional differences between lobular and ductal histology before treatment [312-316]. To our knowledge, there have been no previous studies on the molecular response to endocrine therapy in ILC patient samples. We recently described the clinical response to neoadjuvant letrozole in a series of 61 ILC patients [317] and we and others have characterised the molecular response to endocrine therapy in breast cancer in previous studies [146, 147, 318], but have not considered the effect of histological subtype. Comparing pre- and post-treatment biopsies from the same patients, utilising the 'window of opportunity' afforded with neoadjuvant therapy [319] is a powerful approach which can improve statistical power due to reducing patient-patient variation. However these studies are challenging to perform and are

dependent on analysing sufficient numbers of suitably appropriate samples. In this study we have performed the first gene expression profiling study of ILCs treated with neoadjuvant letrozole and compare the molecular response to that of IDCs.

4.2 Methods

4.2.1 Patients

Samples were selected from a consecutive series of 89 postmenopausal women presenting to the Edinburgh Breast Unit (Western General Hospital) between 2003 and 2011. Each had a large primary histologically confirmed invasive breast cancer, immunohistochemically determined to be oestrogen receptor positive. All patients gave informed consent to be included in the study which was approved by the local Lothian Regional Ethics Committee (LREC; 2001/8/80 and 2001/8/81). Patients were treated within a neoadjuvant protocol in which letrozole (Femara, 2.5mg; Novartis Pharma AG, Basel, Switzerland) was given daily.

4.2.2 Tumour Samples

Tumour biopsies were taken with a 14-guage needle before and approximately 2 weeks (range 10-19 days) and 3 months (range 86-142 days) following commencement of continuous letrozole treatment as described previously, figure 4-1. Samples were snap-frozen in liquid nitrogen and frozen sections taken, stained with haematoxylin and eosin (H&E) and the cellularity and percentage presence of cancerous tissue within each specimen was assessed by a pathologist. Two week samples were available only for 10 of the 14 ILC patients.

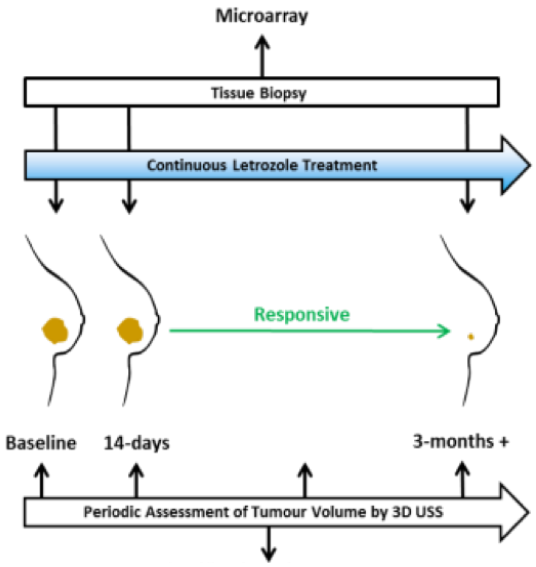

Figure 4-1. Neoadjuvant letrozole in IDC and ILC, study design

4.2.3 Response Assessment

Clinical response was determined using dynamic changes in tumour volumes assessed by repeated measurements taken over the 3 month treatment period. Primary assessment was based on ultrasound measurements performed by a single clinician (JMD) and these were verified by mammographic measurements, figure 4-2. Clinical response was defined as a reduction of greater than 70% in tumour volume by 3 months.

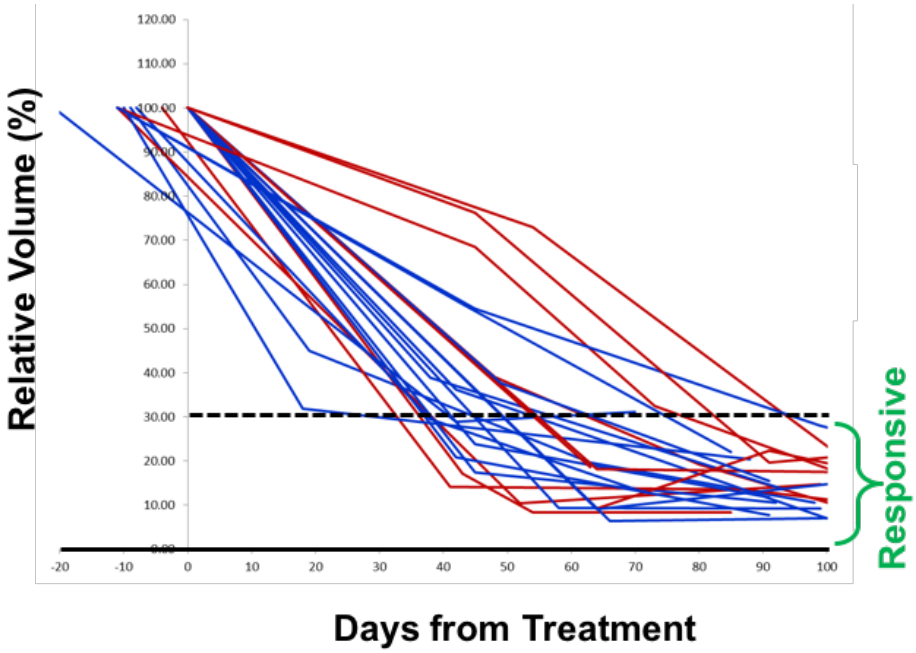

Figure 4-2. Dynamic clinical response to letrozole.
Red – ILC patients, blue – IDC

4.2.4 RNA Processing and Microarray Hybridisation

Biopsies were homogenised and RNA was extracted using the RNeasy Mini Kit with RNAse-free DNAse treatment (Qiagen). RNA quantity and quality was verified on a Bioanalyser 2100 with RNA 6000 Nano Kit (Agilent) and Nanodrop 2000c (Thermo Scientific). RNA was reverse transcribed and amplified using the WT-Ovation FFPE System Version 2 (NuGEN), purified using the Qiaquick PCR Purification Kit (Qiagen), biotinylated using the IL Encore Biotin Module (NuGEN), purified using minElute Reaction Cleanup Kit (Qiagen) and quantified once again using the Nanodrop 2000c (Thermo Scientific). Labelled cDNA was hybridised to Human HT-12v4 whole-genome expression beadarrays (Illumina) according to the standard protocol for NuGEN amplified samples. The Illumina data have been submitted to the NCBI Gene Expression Omnibus (GEO) and are available under GSE55374.

Approximately half of the ILC and IDC samples were processed on Affymetrix GeneChips within a previous study [147], these are publicly available from NCBI GEO under GSE20181.

4.3 Data Analysis

The llumina and Affymetrix data were independently pre-processed and re-annotated to Ensembl gene identifiers, then combined and batch corrected as described previously [257]. Briefly, Illumina probe profiles were quantile normalised using the *lumi* package [258] and mapped to Ensembl gene sequences using reMOAT [259], BioMart and a custom BLAST sequence search. A custom Chip Definition File (CDF) [320] was used to map the Affymetrix data to Ensembl. The Affymetrix portion of data was normalised by robust multi-array average method implemented by the *affy* package. The datasets were filtered using detection P-values, removing probes that were undetected ($p>0.05$ in the total minus 3 samples). Both datasets were then combined and batch corrected with cross-platform normalisation (XPN; ArrayMining) [257, 321, 322]. A subset of samples was profiled on both platforms and demonstrated successful minimisation of batch effects [257]. Pretreatment tumours were assigned to molecular subtypes using the Sorlie and colleagues centroids [43] as described previously [259]. Paired and unpaired Rank Products analysis [323] was used to identify differentially expressed genes and Gene Set Enrichment analysis (GSEA) was performed with the *phenoTest* package. Functional gene ontology analysis was performed using DAVID Bioinformatics Resource 6.7 and the PANTHER classification system. Differences in clinicopathological features and platforms between the ILC and IDC samples were assessed by chi squared test.

4.4 Results

From a cohort of sixty-one patients with ILC treated with neoadjuvant letrozole [317], surgery was possible for 24 cancers after 3 months. Sufficient quality and quantity of RNA for gene expression profiling was available for matched pre-treatment and 3 month samples for 14 of these patients with ILC who had a clinical response to letrozole. Pre- and 3-month letrozole-treated transcriptome data was also selected for a further 14 patients with IDC that responded to letrozole. Patients were matched for clinicopathological features, table 4-1, and response, figure 4-2, and the histopathological status was confirmed by a pathologist, representative images figure 4-3. Consistent with previous studies [312-315], unsupervised hierarchical clustering of the pre-treatment samples using the 500 most variable genes across samples at pre-treatment was able to distinguish between IDC and ILC with 86% accuracy, figure 4-4.

	Lobular breast cancers (14)	Ductal breast cancers (14)	p-val
Grade			0.21
I	0	0	
II	9	13	
III	3	1	
Not available	2		
ER (Allred) score			0.66
7	3	4	
8	11	10	
HER2			1
Negative	13	13	
Positive	1	1	
3-month sample			0.16
Surgical resection	13	11	
Core biopsy	1	3	
Intrinsic Subtype			1
Luminal A	14	14	
Tumour size			0.44
T1	1	0	
T2	7	10	
T3	1	1	
T4	5	3	
Nodes			0.52
Negative	9	8	
Positive	4	6	
Not available	1		
Microarray platform			0.7
Affymetrix	7	8	
Illumina	7	6	

Table 4-1. Clinicopathological features of ILC and IDC patients

No significant differences between cohorts.

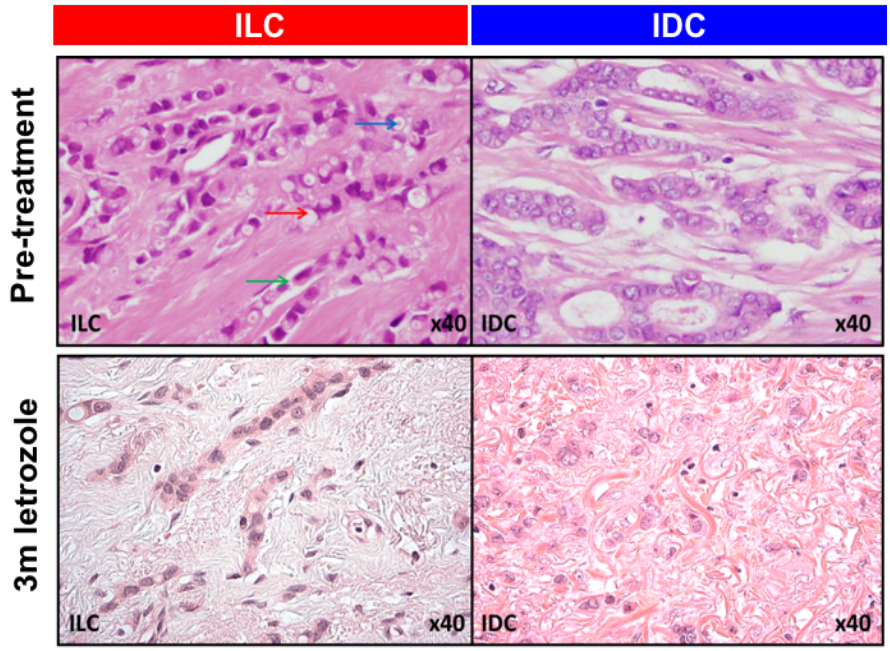

Figure 4-3. Representative images of response to neoadjuvant letrozole in ILC and IDC.

ILC is characterised by cancer cells invading the stroma in single file patterns. Blue arrow, cytoplasmic eosinophilic inclusions; red arrow, vacuolated cytoplasm; and green arrow, the clear spaces between adjacent cells.

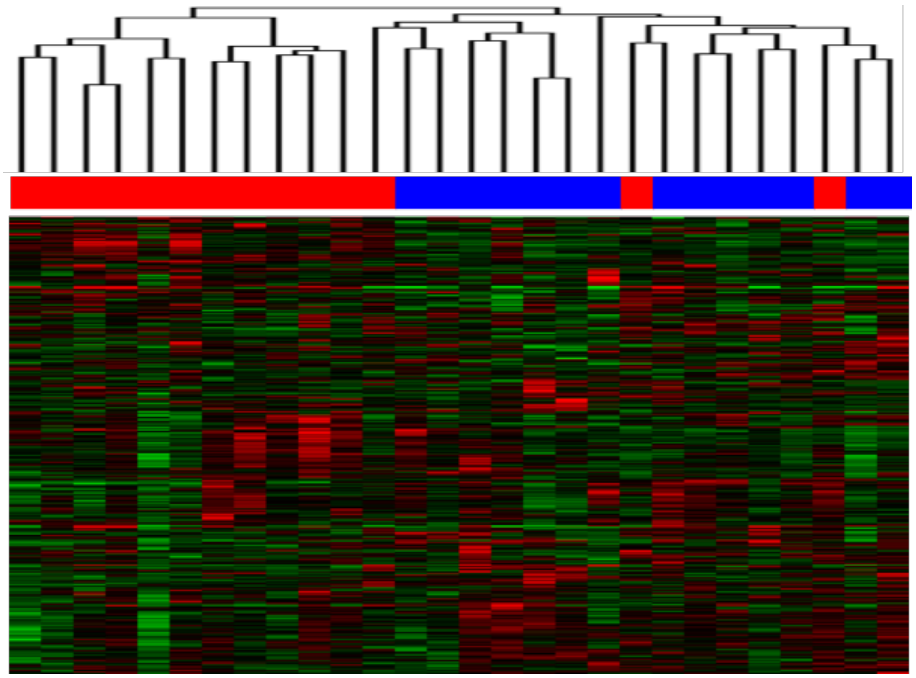

Figure 4-4. Unsupervised HCL of 500 most variable genes in ILC and IDC pre-treatment.

On dendrogram: Red – ILC, blue - IDC.

Heatmap: Red - higher expression, green - lower expression

4.4.1 Molecular differences between ILC and IDC are maintained on treatment

Supervised analysis (Rank Products, percent false present PFP=0.05) identified 206 genes differentially expressed between the histological subtypes prior to treatment. The 70 genes that had lower levels of expression in ILC than IDC tumours, were functionally enriched for immune and extra-cellular matrix (ECM) remodelling genes, including several highlighted in previous studies including e-cadherin (*CDH1*), osteopontin (*SPP1*) and epithelial cell adhesion molecule (*EPCAM*). Similarly, many of the 136 genes with significantly higher expression in ILC than IDC have previously been shown to distinguish between these histologic subtypes [313-316], and include *CAV1*, *AOC3*, *FAB4*, *VWF*, *TF*, *CD36*, *EGR1*, *IER2* and *PLIN1*, figures 4-5 and 4-6. Over half of the genes found to be significantly differentially expressed between the histological subtypes before treatment (including all of those highlighted) were still significantly differentially expressed after 3 months of treatment, figure 4-6. Therefore, differences in gene expression between ductal and lobular carcinomas are maintained during treatment, as illustrated using multidimensional scaling of the 206 pre-treatment genes, with time plotted on the third dimension, figure 4-7.

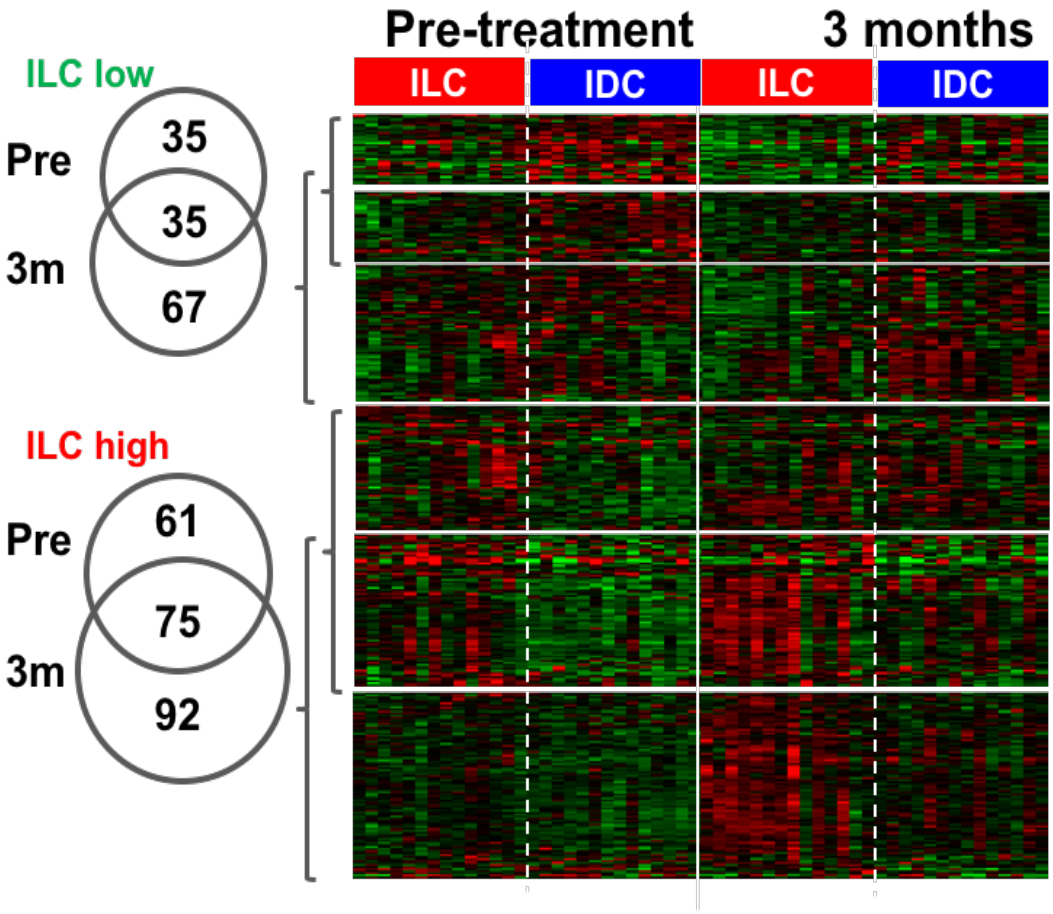

Figure 4-5. Differences between ILC and IDC are maintained on treatment.

Left - Venn diagram shows overlap of genes lower (top) and higher (bottom) in ILC than IDC at pre-treatment and 3 months (3m)

Right - Heat map (RP PFP 0.05) showing changes in same genes between ILC and IDC tumours at baseline and 3 months. Red - higher expression, green - lower expression

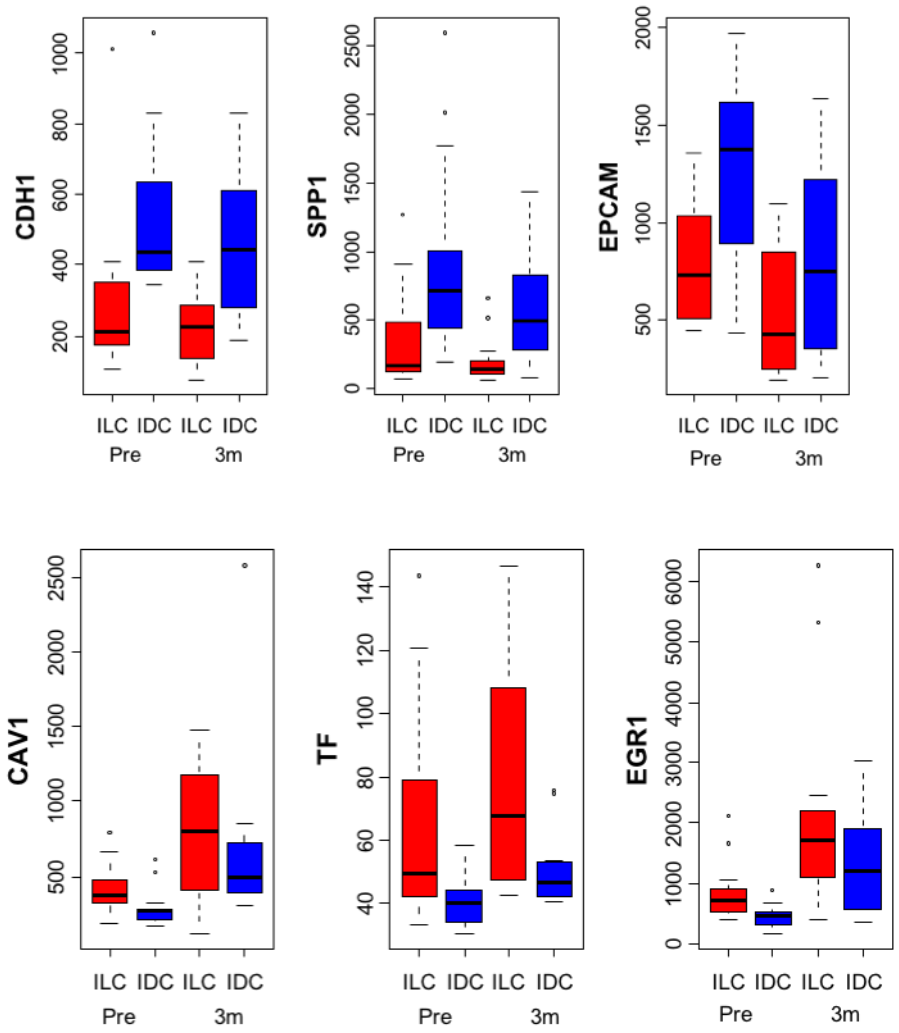

Figure 4-6. Boxplots for genes previously identified as differentially expressed between ILC and IDC

Red – ILC, Blue IDC

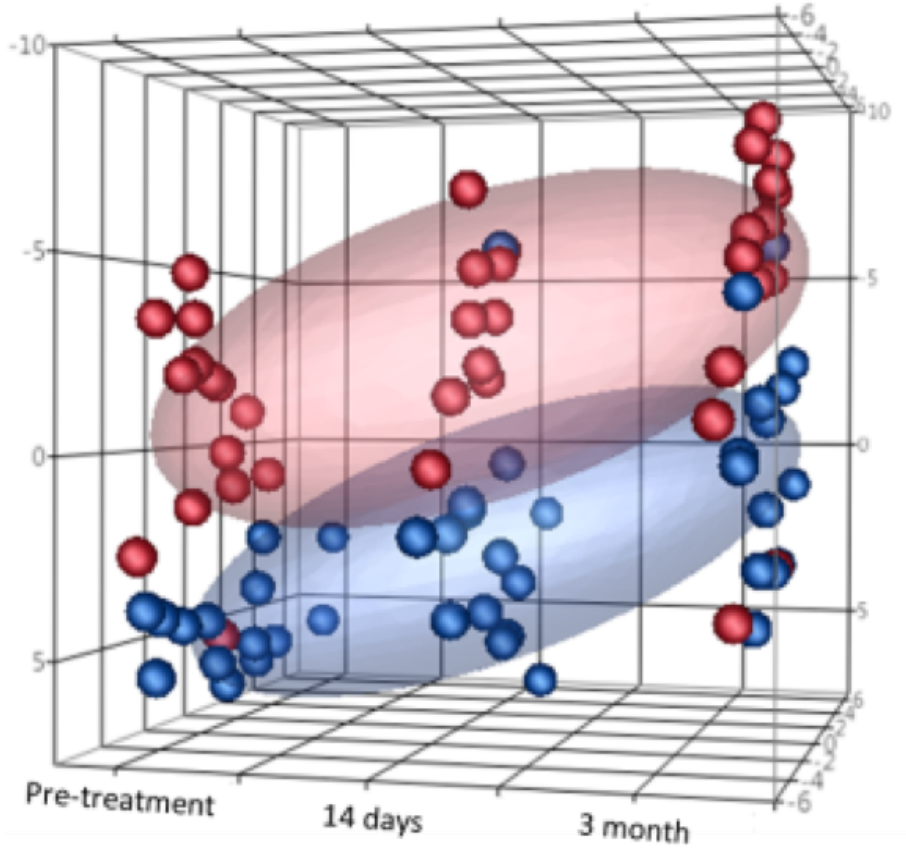

Figure 4-7. Multidimensional scaling plot of 206 genes differentially expressed between ILC and IDC at baseline are maintained on treatment over time
Red – ILC, Blue – IDC

4.4.2 Highly similar molecular response to letrozole in ILC and IDC

Gene expression profiles of surgical samples after 3 months of letrozole treatment were compared with their representative patient-matched pre-treatment biopsy samples using a pairwise Rank Products analysis (PFP=0.05) for ILC and IDC patients. Over half of the changed genes in response to letrozole were significantly up or down regulated in both histological subtypes. Figure 4-8 demonstrates clearly that the molecular effects of treatment are virtually uniform (even after just 2 weeks), in the two subtypes, with the same genes up- and down-regulated.

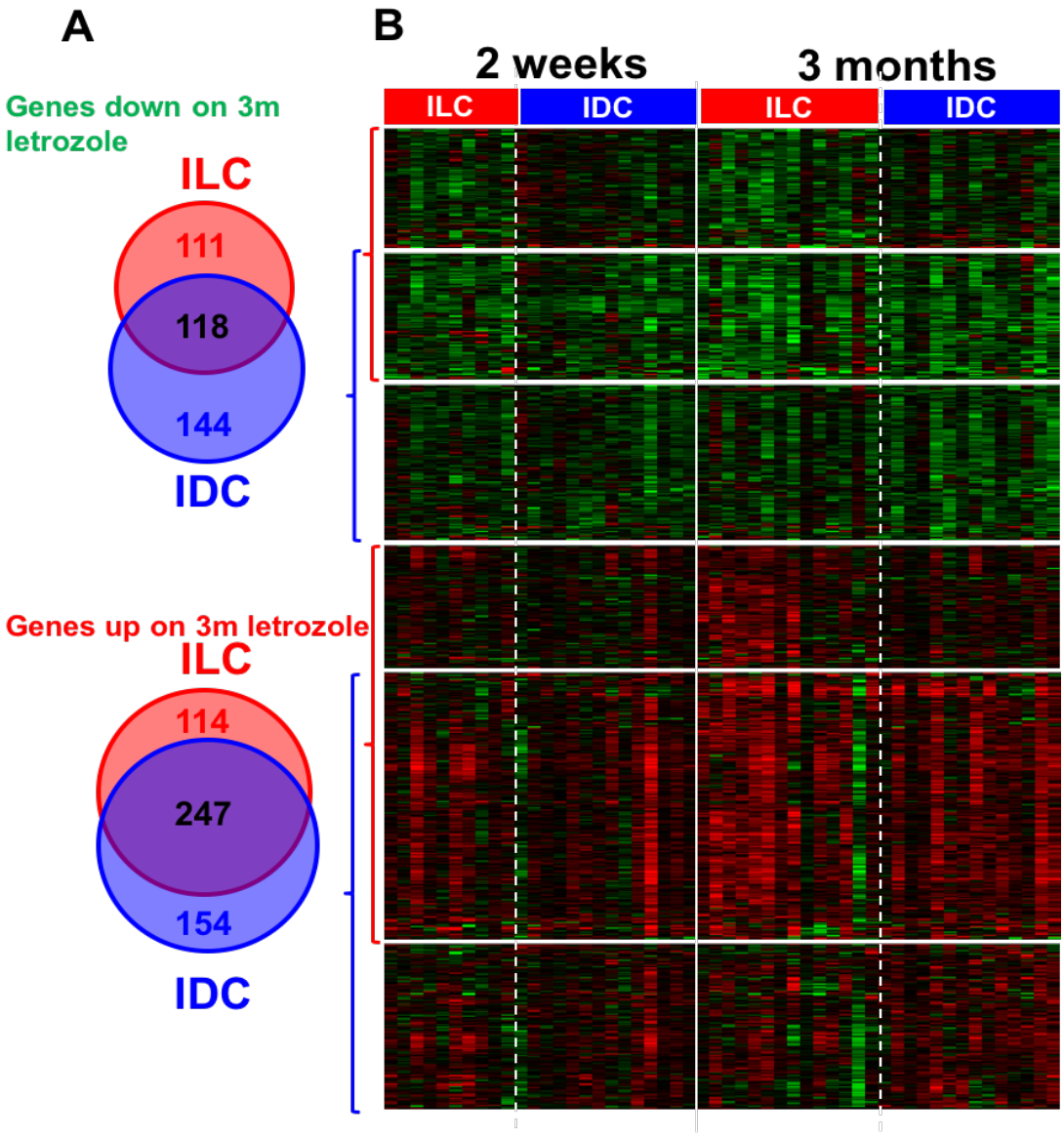

Figure 4-8. Genes differentially expressed between ILC and IDC at pre and 3 month samples

A: Venn diagrams show overlapping genes consistently up- and down-regulated in response to letrozole in ILC and IDC, comparing 3 month and pre-treatment samples by pairwise Rank Products analysis (FDR 0.05).

B: *Heatmap shows genes consistent and specific to ILC or IDC, the colours represent changes in gene expression, red – up, and green down-regulated genes, respectively.*

This result is somewhat at odds with a recent study that found that E2 and anti-oestrogens differentially regulate ERα-mediated gene expression in ILC (MDA-MB-134VI and SUM44PE) and IDC (MCF7, T47D and BT474) cell lines [311]. However, we found that the 'ILC-specific' and 'IDC-specific' genes identified in this study were not significantly changed in the clinical samples after neoadjuvant letrozole, figure 4-9. Gene set enrichment analysis (GSEA) was performed to compare the response between histological subtypes and confirmed consistency of the gene lists before and after treatment ($p<0.0001$), figure 4-10. Furthermore, none of the differentially regulated genes highlighted in ILC cell lines and represented in our data (*CA12*, *NEDD9*, *CXCL12*, *PDE4B* and *NR3C2*) were significantly differently regulated between ILC and IDC tumours treated with letrozole, figure 4-11.

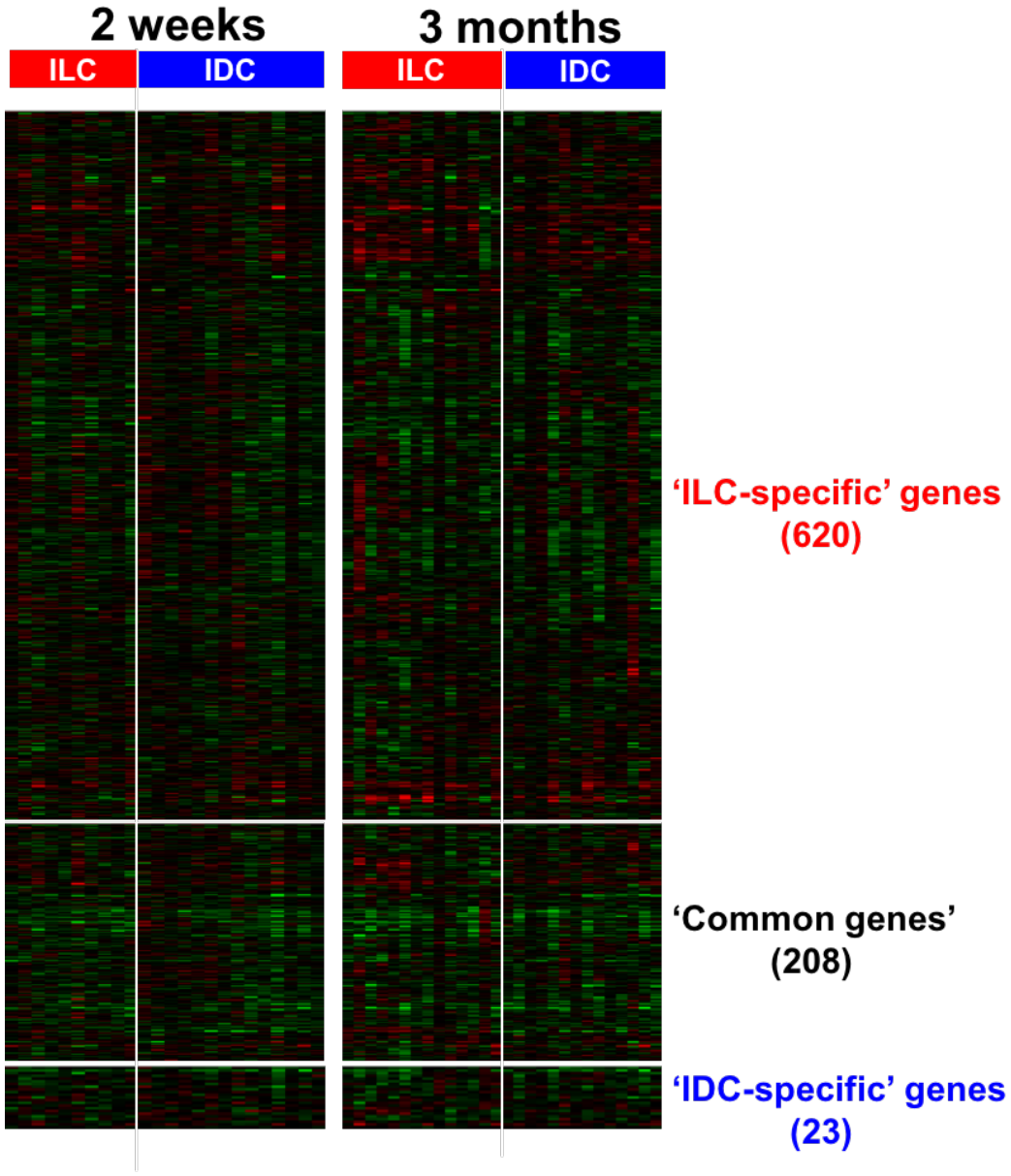

Figure 4-9. Heat map of 'ILC and IDC specific' genes in both groups at 2 weeks and 3 months.

Gene lists previously identified in cell line study [311]. Heat map shows representation of these genes in our clinical letrozole treated samples. Each specific gene list shows very little change over time in response to letrozole in both histological subtypes.

Dendrogram – Red ILC, Blue IDC.

Heatmap – red – higher expression, green – lower expression

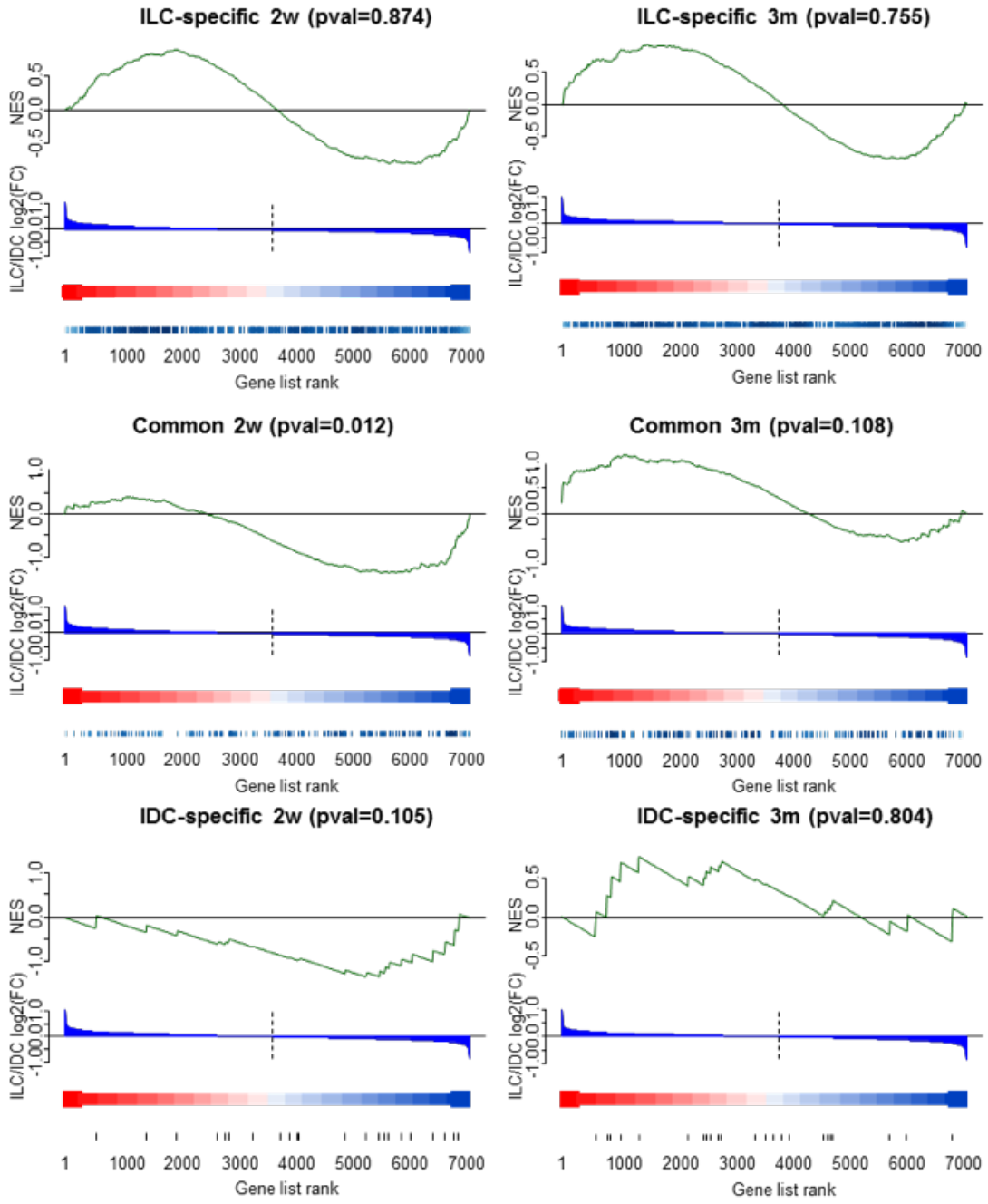

Figure 4-10. Gene Set Enrichment Analysis of ILC-specific, common and IDC-specific gene lists.

Specific gene lists identified in previous cell line study [311]. GSEA shows how these gene lists are represented across all samples in our letrozole-treated patient cohort at 2 weeks and 3 months of treatment. GSEA shows consistency in gene lists between all ILC and IDC samples at both 2 weeks and 3 months showing there is no significant change in these genes in response to letrozole.

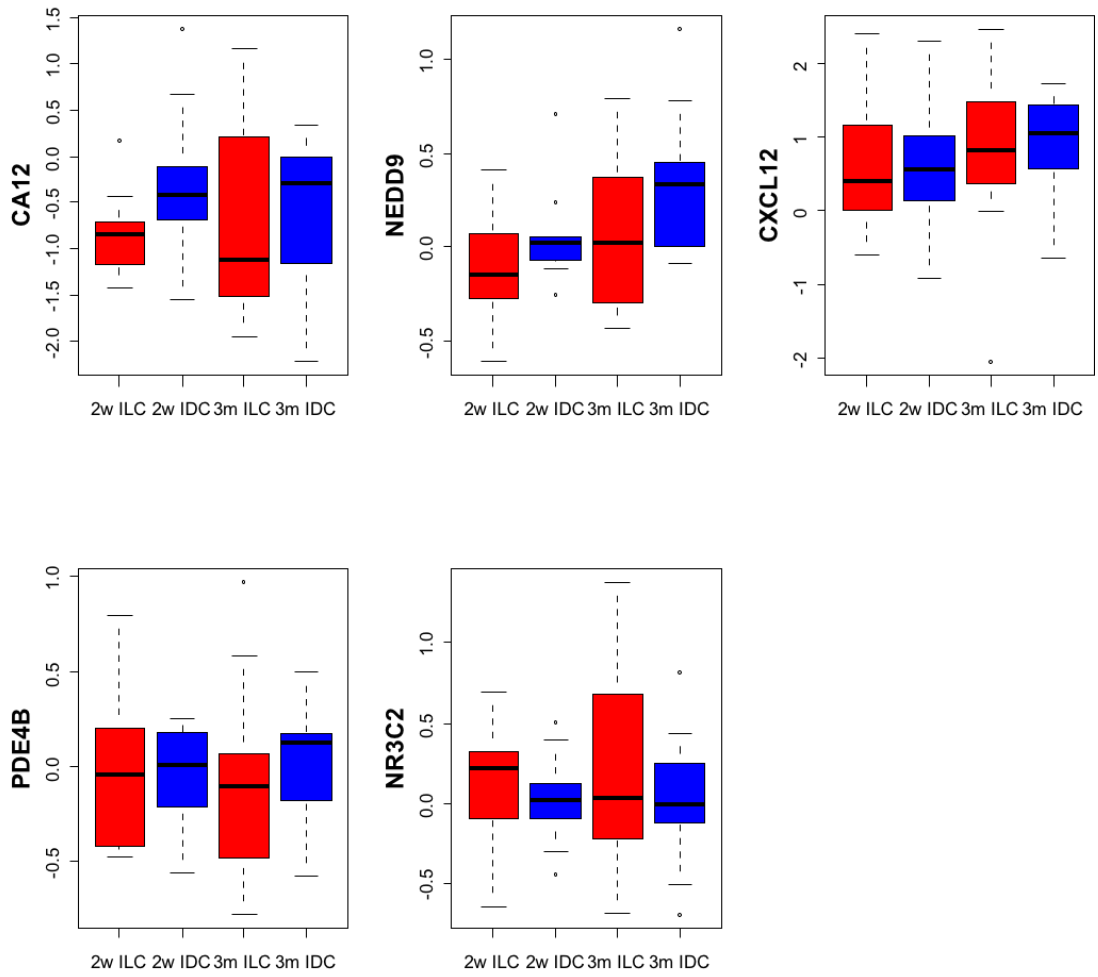

Figure 4-11. Differentially regulated genes from ILC cell line study

Despite being highlighted as differentially regulated in ILC and IDC cell lines, these genes show consistent expression in the patient treated cohort with no significant change in response to letrozole.

Red – ILC, Blue - IDC

Whilst there are distinct genes which differentiate ILC from IDC tumours, these remain constant in response to letrozole and can still differentiate between the 2 histopathological subtypes at different points in treatment. The genes that do change in response to letrozole however are almost uniformly changed across all specimens, independent of histopathological subtype.

Perhaps not surprisingly, and consistent with previous studies [147, 318], the genes that were most significantly changed in response to letrozole in both ILC and IDC tumours were characterised by down-regulation of proliferation and up-regulation of extracellular matrix remodelling pathways, figure 4-12.

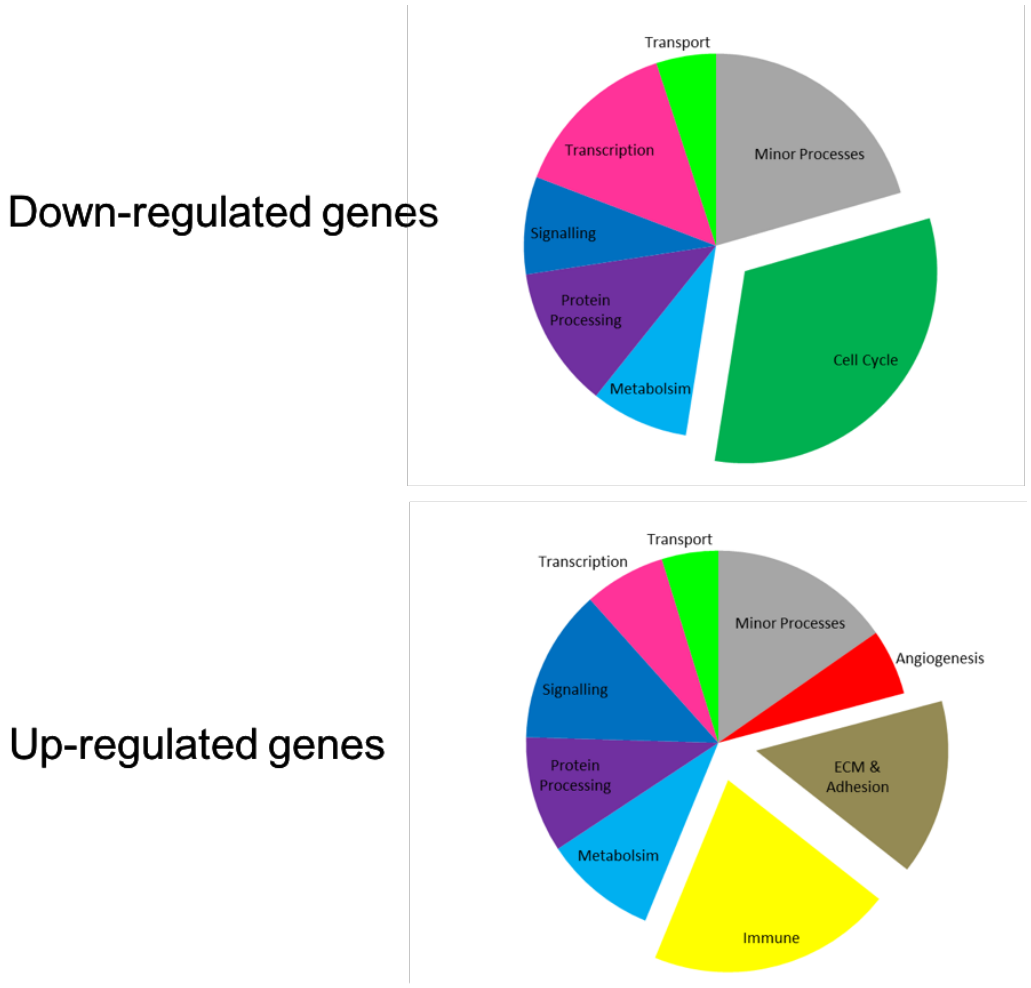

Figure 4-12. Functional association of genes changed with treatment by 3 months Each gene was assigned to only one group which was the most significantly enriched functional process or pathway as determined by DAVID Bioinformatics resource 6.7 and the PANTHER classification system.

4.5 Discussion

Our study shows for the first time that the molecular response to endocrine therapy in ILCs is highly similar to the response in IDCs. This is somewhat surprising given that we and others [313-316], have demonstrated clear molecular differences between tumours prior to treatment. The present study demonstrates that these differences are maintained during treatment. Stratified medicine seeks to identify molecular differences between patients cancers that will allow targeted treatment with specific agents, yet although these histological subtypes of breast cancer are molecularly distinct, both respond in a highly uniform way to endocrine therapy. The consistent molecular changes in expression observed in response to letrozole in both ILC and IDC contrast with a recent study that found that E2 and anti-estrogens differentially regulate ERα-mediated gene expression in ILC and IDC cell lines [311]. Whilst there are obvious possible explanations, including different responses to alternative endocrine agents and the degree to which a small number of cell lines represent the molecular heterogeneity of primary breast tumours, we believe that our study demonstrates the value and need for performing molecular studies in patient samples undergoing treatment, rather than in cell lines. One of the possible reasons behind the similar molecular response to treatment observed in ILC and IDC in this study is that we selected only responsive tumours from both histological subtypes.

Previous work from our group has suggested that there is greater molecular diversity in the gene changes seen between individual non-responding tumours when treated with aromatase inhibitors, whereas in responders the changes are relatively homogeneous [147]. Thus, the resistance mechanisms still may be different between the ILC and IDC despite the similarities found in the present study. This may warrant a further extension of this study focused on the different resistance pathways in non-responding tumours of the different pathological types. However, we have recorded that some non-responding tumours have molecular changes similar to responders; notably reductions in oestrogen-regulated and proliferation genes, without a clear clinical response [148].

Whilst the number of patients in this study is relatively modest, the ILC and IDC patient groups consist of cancers with a consistently high Allred ER score of 7 or 8, all luminal A subtype, grade 2 or 3 and had similar responses to letrozole. High numbers of genes were significantly differentially expressed between the two subtypes, demonstrating clear distinctions between these cancers which was maintained at all time points. The study also supports the potential

value of cross-platform integration of datasets to generate larger datasets with increased power given that clinical samples are relatively scarce.

In conclusion, we have performed the first study of molecular changes in ILC in response to endocrine therapy. The genes which change on letrozole treatment are highly similar in ILC and IDC although clear molecular differences between the histological subtypes are evident between these two cancer types, and these differences are maintained on treatment.

5 Molecular characterisation, subtype concordance and prognostic group assignment between patient-matched primary breast tumours and axillary lymph node metastases

5.1 Introduction

Breast cancer is the most commonly diagnosed female cancer worldwide and the second most common cause of female cancer mortality worldwide. In Scotland it accounts for 12.6% of annual female cancer deaths, around 1,000 women each year [1]. Morbidity and mortality is due to relapsed, drug resistant metastatic disease, rather than the primary tumour. Metastases in regional lymph nodes at the time of primary surgery, have long been recognised as one of the most important prognostic indicators for recurrent disease [324]. In around one quarter of patients, lymph node metastases are present at diagnosis.

Breast cancer is a hugely complex disease spectrum with distinct clinical outcomes. Intra-tumoural heterogeneity is well recognised [325], and many studies have demonstrated discrepancies between expression of biomarkers in primary tumours and paired metastases. This is recognised both when nodal metastases are present simultaneously with the primary tumour [237, 326-328], and also when tumours recur later at distant sites [287, 329-331]. The magnitude of this variability is considerable; with discrepancies in up to 36% of patients in expression of ER, up to 54.2% of PR, and up to 31.6% of HER2, see table 5-1. This could have considerable implications on treatment decisions.

The role of repeat biopsy in metastatic recurrences is becoming increasingly recognised in routine clinical practice [332], as treatment can change in up to one in six women. However, in patients who are node positive at diagnosis, current practice dictates that features of the primary tumour, rather than the lymph node, are used for prognostic profiling, treatment decisions and monitoring response to therapy in the neoadjuvant period [333].

Sites of paired samples	Study	Patients (n)	Discordance rate (%)			
			ER	PR	HER2	Ki67
Primary and synchronous axillary lymph node metastases	Zhao *et al* [328]	54	19.1	11.8	14.8	0
	Tawfik *et al* [327]	103	10	16.9	31.6	18.4
	Li *et al* [326]	107	22.4	17.8	15.9	NR
	Aitken *et al* [237]	211	28.4	23.5	8.9	NR
	Falck *et al* [329]	147	1	16	16	23
Primary and later logo-regional and / or distant metastases	Falck et al [329]	36	13	33	27	18
	Lindstrom *et al* [330]	459	32.4	40.7	14.5	NR
	Thompson *et al* [287]	137	10.2	24.8	2.9	NR
	Sari *et al* [331]	78	36	54.2	14.7	NR

Table 5-1. Discordance in ER, PR, HER2 and Ki67 in primary and paired metastatic breast tumours
NR – not recorded

Although disparity in IHC hormone receptor expression is well recognised between primary and metastatic breast cancer, differences at transcription level are not so well understood. Weigelt *et al* compared gene expression profiling in 7 paired primary and distant metastatic tumours, as well as post mortem samples from multiple sites in five patients, including spinal cord, liver, adrenal, lymph node, lung, kidney, diaphragm, and brain. In unsupervised hierarchical clustering, all samples from the same patient always clustered together, suggesting molecular profiles are maintained in distant metastases [334]. Molecular subtype, as determined by 70-gene signature, was the same in 6 of the 7 paired samples.

Priedigkeit *et al* compared intrinsic subtype by PAM50, in 20 patients with primary breast tumours and brain metastases, and found concordance in 17 (85%). They also found however, loss of *ESR1* in 9 (45%) and gain of *ERBB2* in 7 (35%); with 3 of these 7 also demonstrating new HER2 positivity by IHC in the brain metastases [335]. Despite high concordance overall by subtype, there are obviously huge implications for changes in treatment in up to 45% of this cohort.

Vecchi *et al* found distinct differences in molecular profiles between primary tumours and metastases, and proposed a 4 gene epithelial 'metastatic signature' that could separate primaries from metastases with 70-80% accuracy across 3 cohorts of 115 paired samples [336]. Similarly, Lee *et al* described differences in PAM50 intrinsic subtype between paired primary breast cancers and brain metastases in 8 of 17 patients (47%) [337].

We can improve on traditional clinicopathological estimates of prognosis by assessing tumour biology at transcriptional level [162]. Previous studies have assessed metastases which developed later than the primary tumour. This could reflect changes in response to treatment, rather than innate tumour evolution and progression. This is the largest cohort to date to perform gene expression profile analysis, in paired primary breast tumours and their matched synchronous lymph node metastases.

5.2 Methods

We identified two separate cohorts of patients to participate in this study, see figure 5-1. The first were historic samples from patients operated on between 1999-2002, identified from the South East Scotland Cancer Network (SCAN) Audit Database following approval by both NHS Lothians Tissue Governance Committee and Caldicott Guardians. Criteria for inclusion were primary breast tumours measuring 2cm or more in diameter, with 2 or more positive axillary lymph nodes at the time of resection. FFPE blocks were then retrieved for each patient, and 5μm sections cut for both RNA extraction and to prepare H&E slides.

The second cohort was prospectively collected between November 2012 and January 2014, following approval by the Lothian Regional Ethics Committee. Patients confirmed to have a primary breast cancer with an involved axillary lymph node at diagnosis were included. Core biopsy was taken from both the breast primary tumour (P) and lymph node (N) and snap frozen in liquid nitrogen.

Clinical information for patient demographics and outcomes was collated from medical electronic and paper case note review.

Slides were prepared and stained with H&E from each sample in both cohorts and reviewed with a consultant pathologist. Samples with low cellularity or low tumour content were excluded.

RNA extraction from FFPE tissue was carried out using the Prelude kit (NuGEN) as per the manufactures standard protocol. Briefly, tissue underwent deparaffinisation using xylene and washing with ethanol, then lysis and digestion using proteinase K and binding buffer with β-mercaptoethanol. Preparations then underwent column purification with DNAse treatment and RNA was then eluted in nuclease free water and stored at -80^{o}C until further use.

In fresh frozen biopsies, tissue was homogenised and RNA was extracted using the RNeasy Mini Kit with RNAse-free DNAse treatment (Qiagen), as described previously [338].

All RNA was reverse transcribed to cDNA then amplified using the Ovation WTA System (NuGEN). Amplified cDNA was labelled using the Encore BiotinIL module (NuGEN) purified with the MinElute purification kit (Qiagen) and hybridised to Illumina HT12v4 Whole Genome Expression BeadChips. At each step of the process RNA concentration and purity was assured with Nanodrop 2000c (Thermo Scientific), before progressing to the next stage.

Data was processed and analysed using the statistical programming language R. Illumina probe profiles were quantile normalised using the *lumi* package [258] and mapped to Ensembl gene sequences using reMOAT [259], BioMart and a custom BLAST sequence search. Undetected probes ($p>0.05$) were removed. Cross-platform normalisation (XPN; ArrayMining) [257, 321, 322] was used to correct for batch effects between the FFPE and fresh frozen samples. Unsupervised hierarchical clustering and gene expression heatmaps were generated in MeV; (TM4 Microarray Software Suite) using Euclidean distance with complete linkage following gene mean-centering performed in Cluster 3.0. Intrinsic subtype was determined by proximity to centroid of Sorlie subtype, based on expression of 438 intrinsic gene list [43]. Multidimensional scaling was performed in R with scaling plots generated in JMP10 (JMP Software, USA).

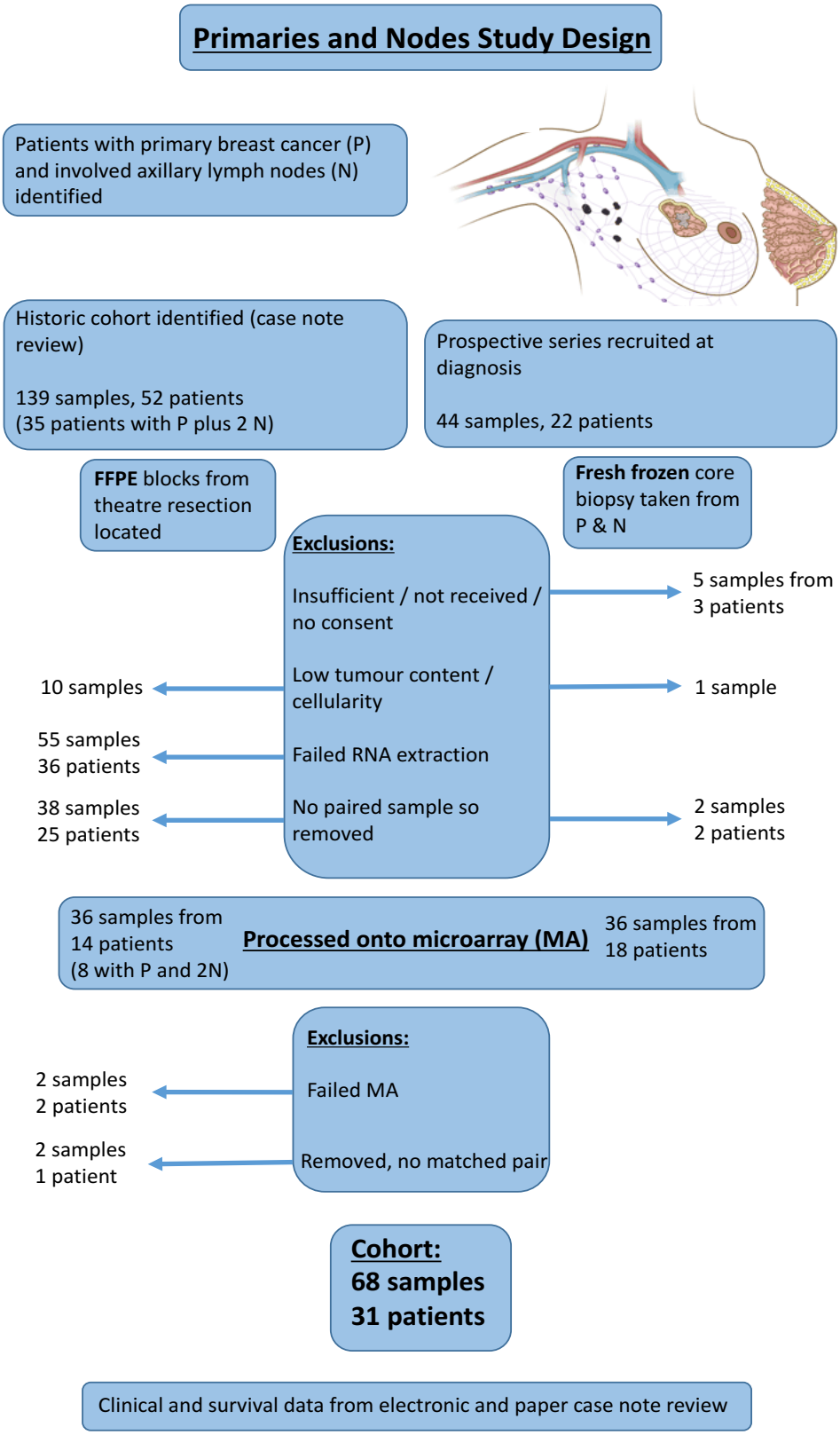

Figure 5-1. Primaries and nodes study cohort

5.3 Results

From the historic cohort 139 samples from 52 patients were identified and we prospectively collected 44 samples from 22 patients. Unfortunately, a large number of FFPE samples failed various stages of RNA processing due to insufficient quantity or quality and had to be excluded. Microarray data was generated for 68 samples from 31 patients in total, figure 5-1.

Clinicopathological features were available for 30 of the 31 patients included on microarray. Multiple features of adverse prognosis were found, not unexpectedly. Most tumours were grade 2 or 3; 53.3% had lymphovascular invasion, and the median number of positive nodes was 6 per patient. The majority (63.3%) underwent mastectomy as the breast surgical procedure, and all patients underwent axillary node clearance rather than axillary node sample or sentinel node biopsy, reflecting the heavy disease burden in the axilla. See table 5-2.

Feature		n	%
Age		Median 61 years Range 40 - 85	
Primary tumour size		Median 25mm Range 13 - 86	
Primary tumour grade	1 2 3	1 16 13	3.3 53.3 43.3
Histology	IDC ILC Other	23 5 2 (apocrine & mixed metaplastic)	76.7 16.7 6.7
Lymphovascular invasion	Yes No	16 14	53.3 46.7
Breast procedure: Mastecomy Breast conservation		 19 11	 63.3 36.7
Axillary procedure Clearance		 30	 100
Primary ER (Allred)	6-8 2-4 0	21 5 4	70 16.7 13.3
HER2	-ve +ve NR	16 3 11	53.3 10 36.7
Number of positive nodes Total excised nodes		Median 6, range 1-20 Median 16.5, range 11-33	

Table 5-2. Characteristics of cohort

5.3.1 Molecular portraits of nodes are distinct from primary tumours

Unsupervised hierarchical clustering of the 500 most variable genes across all samples grouped only 12 of 31 primaries and nodes (39%) together. In most patients, their P or N more resembled a cancer from another patient than its own paired P or N, figure 5-2.

Interestingly however, 6 patients had 1 P matched with 2 N samples. In 2 of these, all 3 clustered together, and in a third, both nodes clustered together, albeit separate from the primary. This implies a gene signature common to lymph nodes in these patients.

Overall the P and N samples trended to clustering separately; orange N towards the left of this heatmap, pink P samples towards right. This may be due to distinct genes related to site of origin of each tumour biopsy.

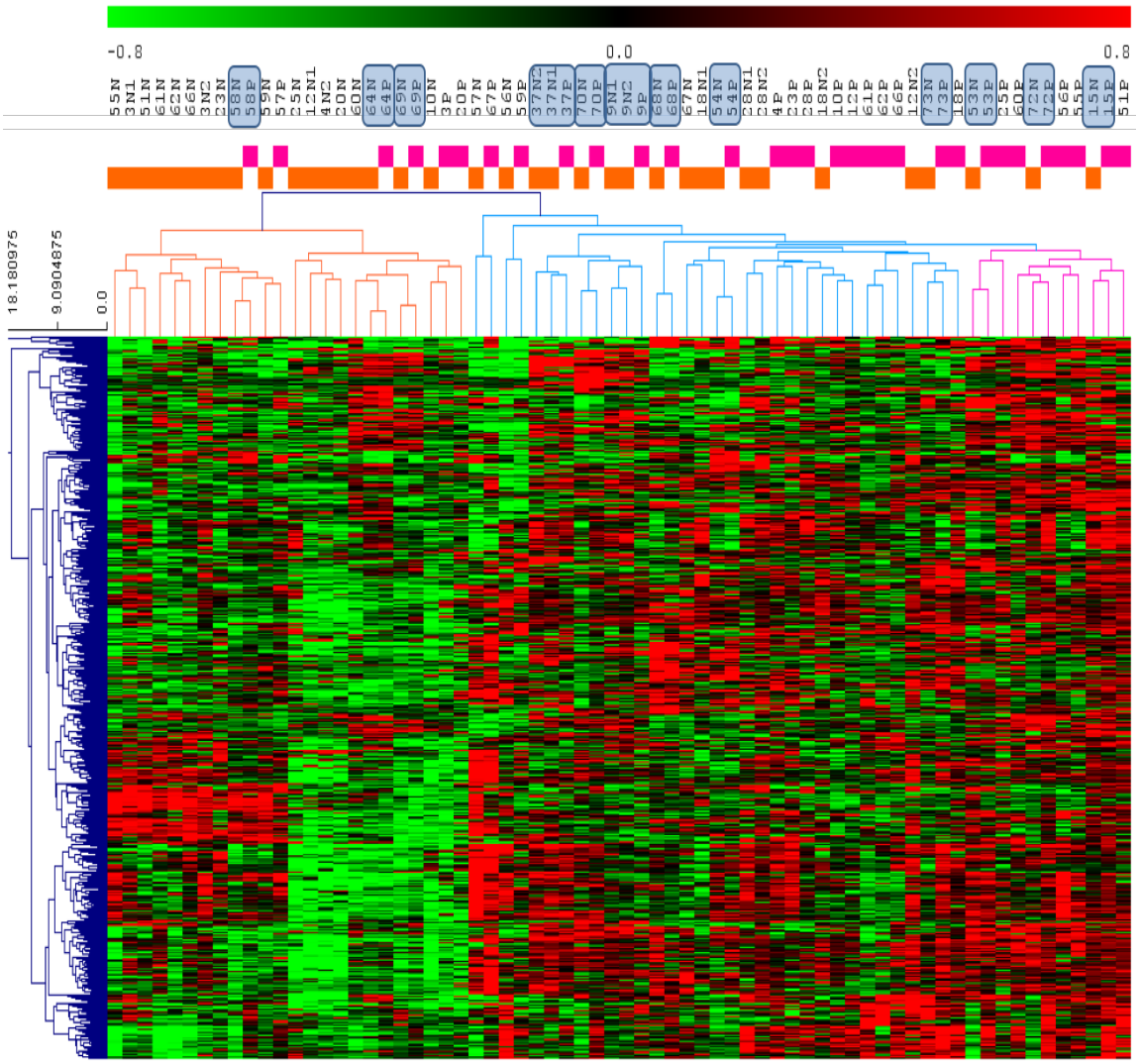

Figure 5-2. Unsupervised HCL of 500 most variable genes in all samples

Pink – primaries, Orange – nodes.

Patients where primary and node cluster together are highlighted.

5.3.2 Changes between primary tumours and nodes vary between patients

The number of genes with greater than 2-fold change (>2FC) between P&N was used to categorise paired samples into 'least changed' (<130 genes with >2 FC) and 'most changed' (>370 genes with >2FC) groups, figure 5-3.

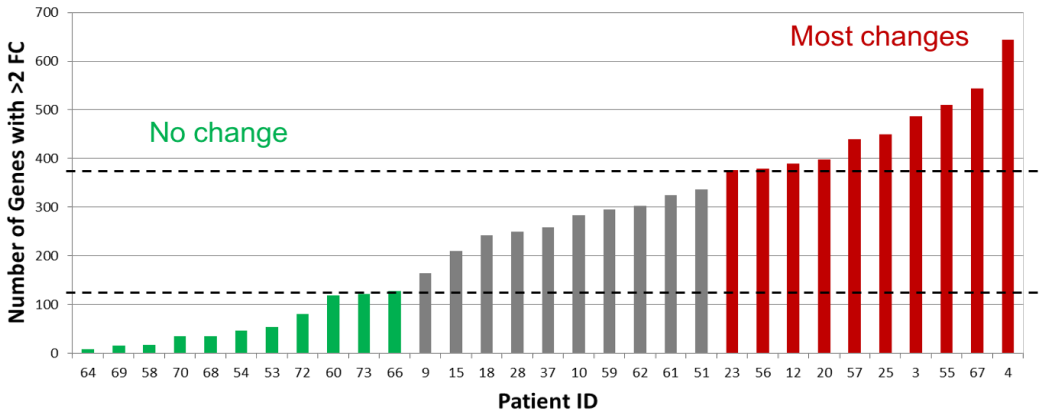

Figure 5-3. Number of genes per patient with >2-fold change between primary and node

In the 10 most changed tumours, multidimensional scaling of the 500 most variable genes showed consistently that nodal metastases differed molecularly from the primary cancer.

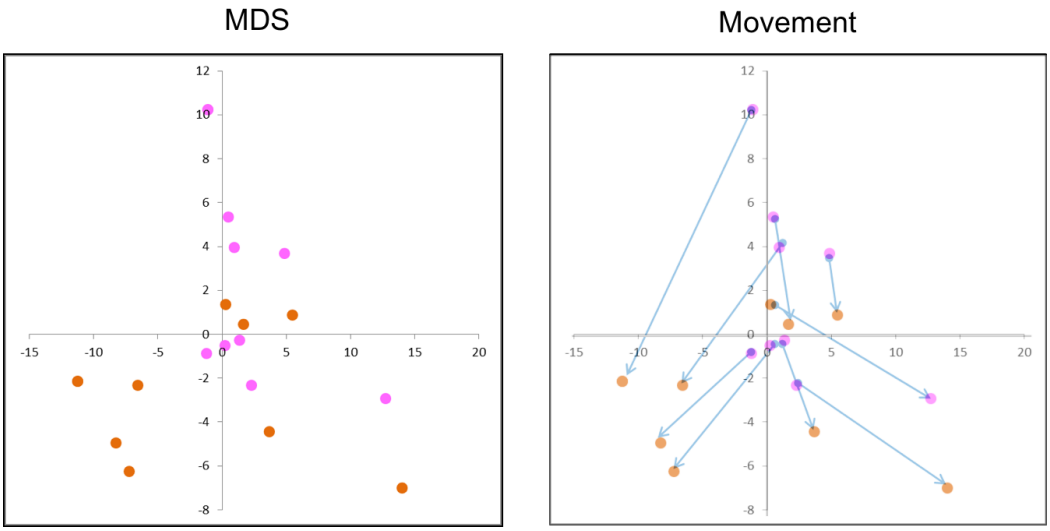

Figure 5-4. Multidimensional scale of 500 most variable genes in most changed samples

Pink – primary, orange – node, MDS – multidimensional scaling plot

Left shows distribution of all samples. Right shows matched samples from same patient demonstrating consistent changes between P&N.

Across all samples, again consistent changes between matched P&Ns were seen, figure 5-5.

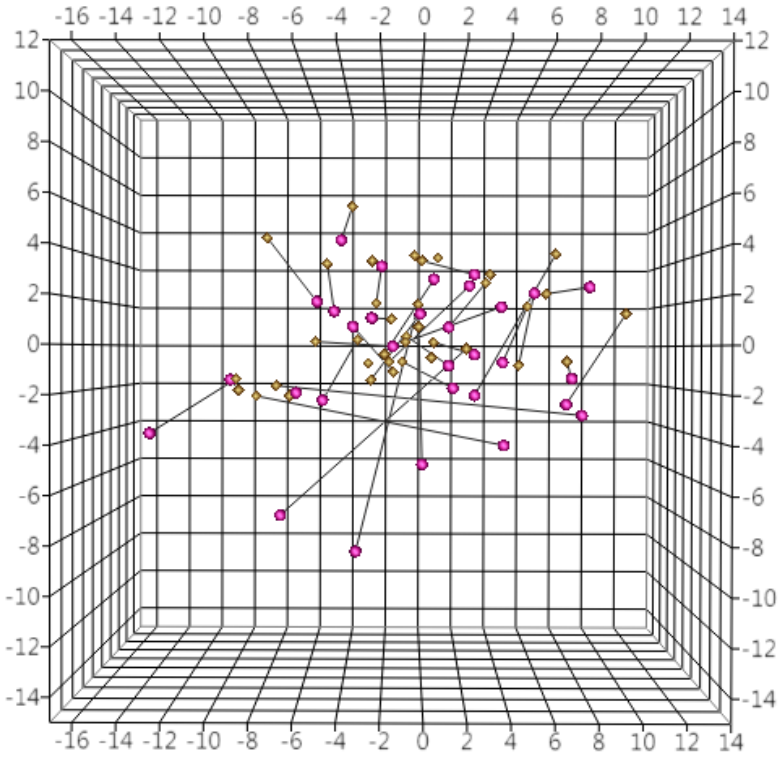

Figure 5-5. Multidimensional scaling plot of all samples based on expression of 500 most variable genes.
Pink circle – primary, orange square - node;
lines link matched P and N from same patient

5.3.3 Intrinsic subtype is not maintained in primary tumour and nodal metastases

When categorised by Sorlie subtype, most primary tumours were luminal A (15 of 31, 48.4%), followed by luminal B (11 of 31, 35.5%).

In the nodes, most were luminal B (22 of 37, 59.5%), followed by luminal A (13 of 37, 35.1%).

A larger proportion of nodal metastases became luminal B than any other subtype, however this did not reach significance (p=0.77), figure 5-6.

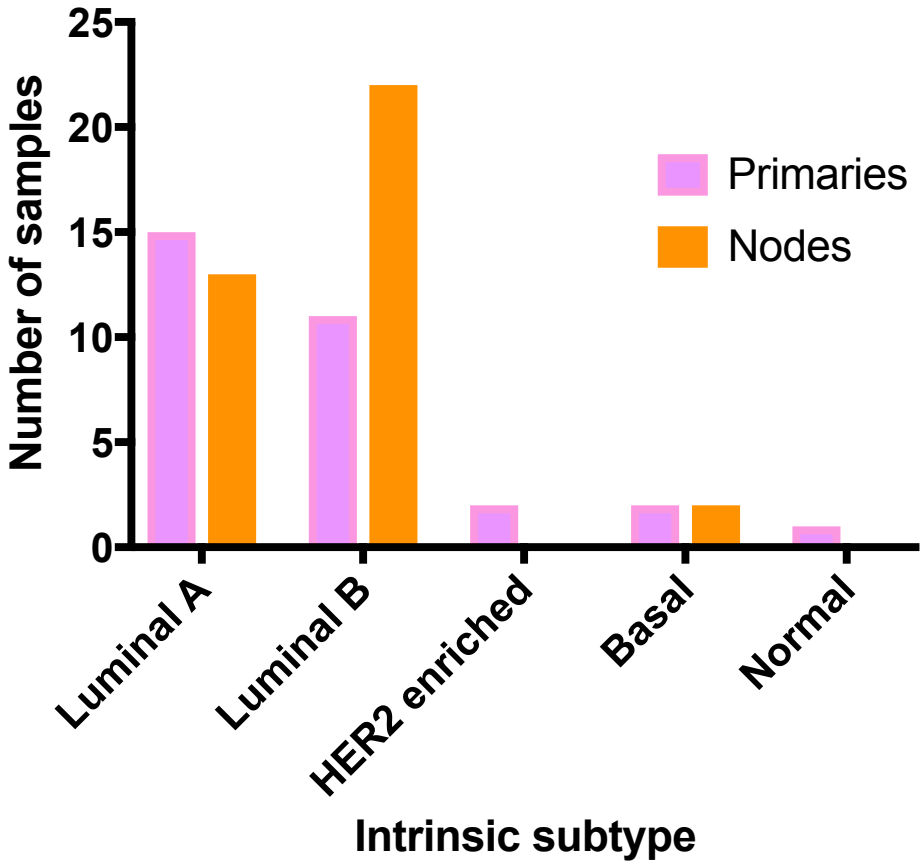

Figure 5-6. Distribution of primaries and nodes by intrinsic subtype across all samples

In paired samples 12 of 31 patients (39%) had a different molecular subtype in the node compared with the primary. In changed samples, the node tended to be a poorer prognostic subtype than the primary, see figure 5-7. In 6 of the 12 which changed subtype (50%), a luminal A primary became a luminal B node. The remaining 50% changed in other non-consistent patterns.

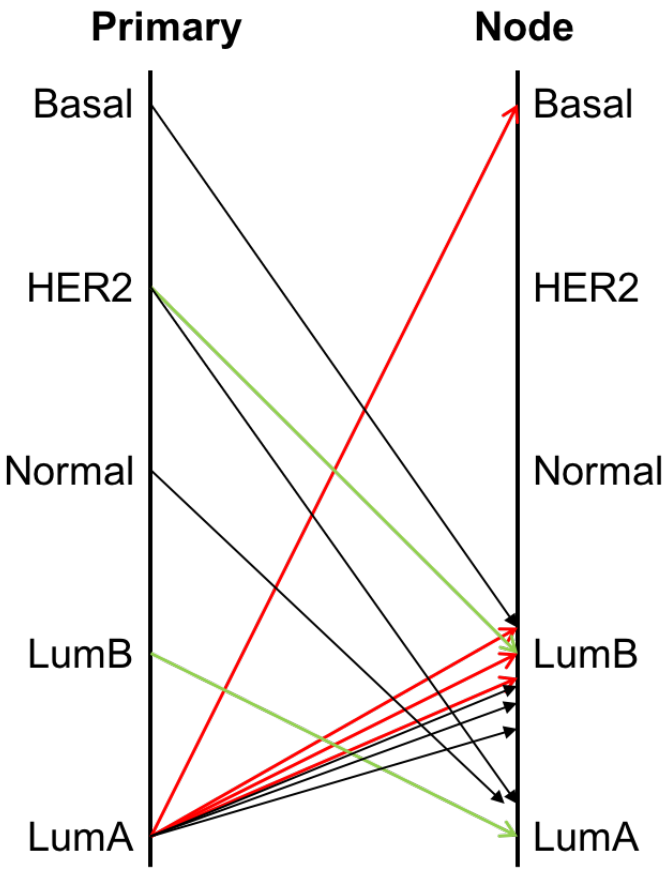

Figure 5-7. Change in subtype between primary and node

Red lines denote patients in 'most changed' group (>370 genes with >2FC between P&N)

Green lines denote patients in 'least changed' group (<130 genes with >2FC between P&N)

In 6 patients with one primary and 2 matched nodes, 2 tumours changed subtype, shown in figure 5-8. In the other 4 patients, subtype remained consistent across all 3 samples.

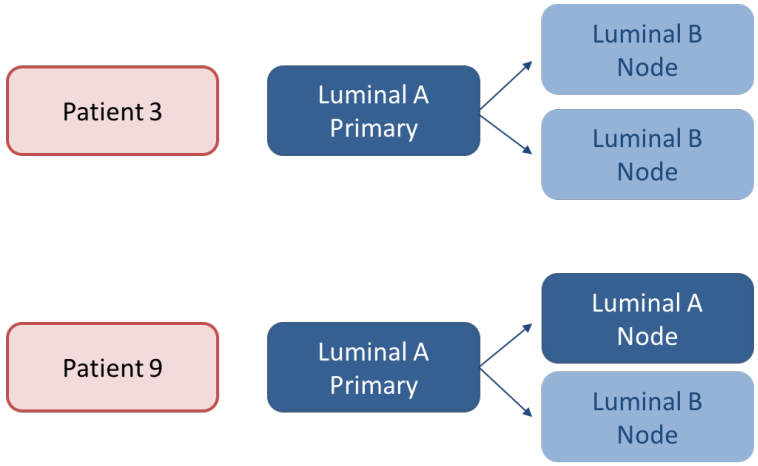

Figure 5-8. Change in subtype in 2 patients with P+2N

5.3.4 Changed tumours and survival

Progression free and overall survival was compared in the 'least changed' and 'most changed' groups. However, there was a lack of events in the 'least changed' group, all of whom originated from the prospectively collected cohort, therefore no comparison was possible. Follow up period in this cohort was inevitably much shorter than the historic cohort (prospective - median 356 days, range 146-551; historic - median 4,695 days, range 4,458-4,911 days).

Of the 13 patients in the historic cohort, survival data was available for 12.

There were 9 recurrence events, with a median progression free survival of 959 days (range 0-4,676 days). There were 8 breast cancer specific deaths, at a median of 1,373.5 days (range 582 – 1,833 days).

5.3.5 Expression of *ESR1*, *PGR* and *ERBB2*

Mean level of log2 expression across all samples was calculated for *ESR1*, *PGR* and *ERBB2*. A single sample within a pair, with expression outwith 2 standard deviations of the mean was highlighted as a discordance.

Discordance rates between P and N in all patients were:

ESR1 - 10 of 31 (32.3%); loss in 3, gain in 6, 1 patient with 2 N – 1 gain, 1 loss,

PGR - 6 of 31 (19.4%); loss in 3, gain in 3,

ERBB2 - 5 of 31 (16.1%); loss in 3, gain in 2.

5.4 Discussion

We have demonstrated metastatic axillary lymph nodes are distinct from their matched primary in up to 39% of patients. The number of genes changed in the node compared to the primary, varies markedly between patients. However, even in those with fewer numbers of genes changed, there can be discrepancy in intrinsic subtype. Metastases tend to be a poorer prognostic subgroup than their primary. Tumour biology is established as a marker of prognosis and recent guidelines have recommended assessment of molecular subtype as a routine of care [339].

Previous studies have focused on discrepancy in subtype in metastases developed at a later date. We have shown however, there is discordance even at the time of diagnosis, and primary surgery in node-positive disease. There was no neoadjuvant therapy given to this cohort, which removes the influence of drug therapies as a cause of genomic aberrations, proving these changes are due solely to tumour evolution and progression.

Our findings support the study by Vecchi which demonstrated synchronous lymph nodes were distinct from their matched primary [336]. Similarly, our results are comparable to the Lee study, where discordance in PAM50 subtype between paired primary breast and brain metastases was 47.1% (8 of 17). Furthermore, 5 of 6 patients in that study, with luminal A breast tumours, changed subtype in the brain metastases (three to HER2, two to luminal B) [337]. This parallels the direction of change observed in our cohort.

Our results contrast however, with previous autopsy studies where tumour specimens from multiple metastatic sites in the same patient always clustered together [334, 340]. Weigelt also profiled metastatic axillary lymph nodes with paired primaries alongside the autopsy tissue; and found 6 of 8 primaries and nodes paired together, which is less concordant than the primaries and distant metastases [334]. This may reflect organ specific heterogeneity in gene expression. In our study, nodes and primaries trended towards opposite ends of the dendrogram. Gene signatures predictive of metastatic potential to particular distant sites have been proposed. Lee described a 5-gene signature which could distinguish brain metastases from breast primaries [337]. Szekely *et al* reported frequent loss of hormone receptors in lung and liver metastases (10 of 16 patients) but not at other sites in their autopsy study of 25 patients with multiple metastases profiled (up to 10 per patient). They defined St Gallen subtype by IHC, and found subtype discordance between primary and metastases in 52% (13 of 25) [341]. Discordance between multiple samples of metastases from the same site however,

was lower. They also observed the most frequent change was luminal A primary to luminal B metastases.

Flack *et al* also assessed subtype by St Gallen IHC criteria in paired primary and synchronous lymph nodes. Discordance in receptor status was: ER 1%, PR 16%, HER2 16% and Ki67 23%, but there was no significant discordance in overall St Gallen subtype [329]. In 15 patients where subtype changed from luminal A primary to non-luminal A node, mortality was comparable to patients with non-luminal A stable disease both in primary and node. Similarly, in 13 patients with non-luminal A primary and luminal A node, mortality risk was less than patients with stable non-luminal A disease at both sites. Although these are very small numbers, achieving luminal A in nodal metastases seems to confer better prognosis. One can postulate nodal subtype may have more clinical relevance in prediction of prognosis than primary tumours in node positive disease.

There are limitations of our study however, which must be considered. This included 2 quite distinct cohorts, one of which was selected from historic records and was biased for heavy burden disease; both in terms of number of positive nodes, and primary tumour size. The quality and quantity of RNA extracted from the historic FFPE tissue was markedly poorer than the prospective frozen cohort, another source of bias. The initial planned cohort was subsequently substantially reduced, therefore no survival analysis was feasible.

Nonetheless our findings have immense clinical significance, with implications in treatment of node positive disease in the neoadjuvant and adjuvant setting. In our cohort, 3 patients lost ER and 3 lost HER2 between primary and node. Current practice dictates they would endure adjuvant targeted endocrine and HER2 therapies, however had the node been profiled treatment would be markedly different. Increasingly we are recognising the heterogeneity of late recurrence and the need to re-biopsy rather than base treatment on historic tissue [287]. Despite this, synchronously resected involved metastatic lymph nodes are not routinely analysed for hormone receptors or biomarkers, therefore have no influence on treatment plans or predicting prognosis.

5.5 Conclusions

Involved metastatic lymph nodes at diagnosis impact on survival. Classifying cancer molecular phenotype and estimating prognosis based only on the primary cancer misclassifies

significant numbers of patients. Classification of prognosis, and treatment based on the nodal metastasis, may provide better information on which to base treatment.

6 Overall Conclusions and Perspectives

The results presented in this thesis illustrate overwhelmingly the enormous value of assessing sequential repeat biopsies in breast cancer patients at different time points in their disease. Breast cancer is a hugely complex and constantly evolving process. High throughput technologies have proven invaluable in advancing our understanding of its intricacies. The work in this thesis has made valuable contributions to our understanding of breast cancer, and also poses significant and important questions which are applicable in routine patient care.

6.1 Validation of on-treatment biopsy

In the non-treated cohort, most tumours (18 of 25) clustered together at baseline and at second sample. This confirms the individual molecular portrait of early breast cancers is maintained over time, when no treatment is given. Neoadjuvant treatment downstages large primary tumours [71] and increases rates of breast conservation [138]. It also allows assessment of molecular response to drugs, with prediction of long-term response to endocrine therapy feasible after just 2 weeks [149]. In letrozole treated ILC and IDC tumours, a distinct gene signature at baseline differentiated them from each other, and was maintained over the treatment period. The global gene changes in response to letrozole however, were almost uniform in both.

In our non-treated cohort, there was little overlap (19%) in the genes changed compared with letrozole treated tumours. This affirms changes in treated tumours are truly due to drug effects. Furthermore, on treatment measure of biomarkers, including Ki67, are more prognostic and predictive of response than they are at baseline, validated first in IMPACT [30], and more recently in Z1031B [193]. This non-treated cohort validates the use of a second biopsy, to assess on-treatment biomarkers, such as changes in proliferation, confirming changes are truly drug related, rather than reflective of tumour evolution.

6.1.1 Surgical specimens, an underutilised resource?

We have highlighted a minority of patients however (7 of 25), where there were changes between the core biopsy and surgical specimen, in whom sequential samples did not cluster together. Others have also described changes in small numbers of patients, between core biopsy and surgical specimen with no intervening treatment [247] resulting in discordance in subtype assignment also [248]. This of course has major implications for adjuvant treatment.

Currently we use the core biopsy to determine ER, PR and HER2. We have affirmed that the core biopsy is truly reflective of overall tumour biology, however intra-tumoural heterogeneity is renowned. An alternative approach would be assessment of sequential biopsy over time, which may offer a more realistic representation of the representative inherent heterogeneity and tumour biology. Reanalysing biomarkers at surgical resection, if introduced into routine care, could highlight changes inherent to natural tumour evolution over time, and identify high-risk patients early.

6.1.2 Use of routine peri-operative treatment

In IMPACT, 52 of 56 (93%) patients showed some suppression of proliferation with 2-weeks of anastrozole [30], suggesting that nearly all ER+ patients derive some benefit from aromatase inhibitor treatment. The use of short course pre-surgical and peri-operative endocrine treatment is becoming increasingly routine. POETIC will assess long-term outcome in patients who received peri-operative AI [194] in due course. This highlights an exciting opportunity to assess repeat sample after short course treatment which could be incorporated into routine practice, to provide a more reliable estimate of prognosis. Furthermore, this could address important questions prospectively for adjuvant treatment.

6.1.3 Implications for adjuvant treatment

Sequential and combination drug therapy approaches have proven effective in the management of metastatic ER+ breast cancer. Everolimus [218], fulvestrant [110], and palbociclib [233] given with AIs, have restored endocrine sensitivity and improved progression free survival. The success in the metastatic setting has prompted phase III trials to explore their use in the neoadjuvant period. ALTERNATE and NeoMONARCH will assess neoadjuvant fulvestrant and abemaciclib respectively, given alone or in combination, with anastrozole over anastrozole alone, to assess clinical outcomes and on-treatment Ki67. NeoMONARCH has shown greater interim Ki67 reduction with abemaciclib, but survival data is yet awaited [196].

If neoadjuvant or perioperative treatment with one agent proves ineffective, this could be recognised at the time of surgery, from the resection specimen. This provides the option to switch to an alternative endocrine agent, or to add a second targeted drug, such as a CDK4/6 inhibitor or mTOR inhibitor, in the adjuvant period, in order to improve outcome. This would have enormous potential as it could identify earlier, patients in whom standard endocrine therapy is insufficient, and alternative agents could be employed, sparing them from ineffective treatment.

6.2 Molecular changes in metastases

6.2.1 *PIK3CA* mutation status is stable in metastases

PIK3CA status was largely maintained between primary tumour and secondary event with change in only 14 of 89 patients (15.7%). Change was significantly more likely in a second new primary breast cancer rather than any other secondary event. This does not support the theory that *PIK3CA* mutation is gained as a mechanism of resistance, unlike evidence for *ESR1* [220]. In BELLE2, *PIK3CA* mutation was a biomarker of response to buparlisib [219]. Our findings confirm assessment is possible from archived FFPE samples and is consistent over time, so diagnostic mutation status could be used as a biomarker in later years. In the future serial assessment of ctDNA may prove useful in this role, however further validation and standardised measures will be required before this is available in the clinic [342].

6.2.2 Nodal metastases are distinct from primary tumours

A significant proportion (12 of 31, 39%) of patients had discordance in molecular subtype between nodal metastases and their matched primary, most commonly becoming more poorly prognostic. Similar changes have been demonstrated in asynchronous brain metastases previously [335, 336] but the findings demonstrated herein, confirm these changes at diagnosis and at primary surgery in node positive patients. Classifying molecular phenotype and estimating prognosis based only on the primary tumour could misclassify significant numbers of patients and potentially offer ineffective treatments, or deny patients from the most appropriate options. Most node positive patients would be considered for neoadjuvant chemotherapy. This presents another opportunity for consideration in the future where primaries and nodes pre and post neoadjuvant treatment could be assessed.

6.3 Limitations and future directions

A major limitation and frustration during this project was the rate of success in using FFPE tissue for RNA microarray analysis from the primaries and nodes cohort. The initial 139 specimens reduced eventually to 36 after poor yield during RNA extraction and subsequent exclusion of further samples because their matched pair was no longer available. The change in subtype is a potentially major clinical finding however, it lacks significance as the small numbers precluded any valuable survival analysis. Consequently, we have not confirmed that the subtype changes observed are of clinical relevance. Nonetheless it highlights a very important observation which is worthy of further investigation.

Furthermore, gene expression profiling is costly and not routinely available. IHC or qRT-PCR however are more routinely available and could be employed at serial biopsy, whether this be in positive node or resected surgical primary. Assessments of molecular subtype and prognosis are possible through IHC and qRT-PCR indices [149, 169, 170, 172, 188]. This may be helpful in providing a assessment of tumour biology. However, commercially available assays, including OncotypeDx and PAM50 are only offered through centralised laboratories to minimise error.

Future work will also involve assessment of alternative RNA extraction and processing methods specific to FFPE tissue [343]. FFPE blocks are readily available and archived tissue could represent an amazing resource to assess retrospective biomarkers in specific cohorts. Expansion of the primaries and nodes cohort with further historic samples will be explored and compared with survival data to determine the prognostic significance of subtype discordance.

A further option to be explored is combining data from these cohorts with large publicly available datasets of samples with comparable cohorts [257]. This may help to validate, or indeed may refute, the differences observed here, including change between core biopsy and resection, and subtype discordance between primary and node.

6.3.1 Improving estimates of prognosis and prediction to treatment

There is a need to distinguish high risk luminal disease from those with excellent prognosis to stratify chemotherapy combined with endocrine therapy, over endocrine therapy alone [188], in both adjuvant and neoadjuvant treatment. TAILORx identified low risk ER+ node negative patients who could forego adjuvant chemotherapy [176] with similar evidence from the use of PAM50ROR, where a subgroup of ER+ node negative patients had >95% survival without adjuvant chemotherapy [179]. There is disagreement between clinicopathological and molecular assessment of risk frequently. This presents a clinical dilemma, and an area where further research is necessary. RASTER demonstrated discordance between 70-gene signature and Adjuvant! Online, St Gallen classification and NPI in 37%, 39% and 27% respectively [185]. MINDACT also confirmed discordance between clinical and genomic estimation of risk but was underpowered to confirm benefit of chemotherapy in the discordant groups [187]. Further evaluation is also required in patients deemed intermediate risk in genomic scoring. Combining clinical features and biological biomarkers is an emerging option, with evidence of superiority over clinical features or biological markers alone [179, 189]. A Chemo-

Endocrine Score has been proposed, where a gene signature on treatment is predictive of endocrine sensitivity and of chemotherapy resistance. This can improve on ROR score and will help to further evaluate best management of intermediate risk score patients [344].

A further emerging strategy is the role of the liquid biopsy. Early evidence exists for its utility as a biomarker in metastatic disease [345]. Recently it has also shown promise in the adjuvant setting [342]. This could revolutionise care for patients, with prediction of recurrence before any measurable disease burden has emerged, potentially.

We have affirmed molecular changes in both treated and non-treated samples; and both discordances and similarities between different cohorts of primaries and metastatic biopsies. This reflects the complex evolution of ER+ breast cancers. This project has reinforced the benefits of the neoadjuvant period as an opportunity to explore biomarkers. Additionally, we have further corroborated the advantage of profiling matched patient samples over time, treatment and at different sites of disease, as a powerful opportunity to explore biomarkers and estimates of prognosis, in a strive towards personalised medicine.

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8 Supplementary

8.1 Proliferation genes are un-changed in non-treated samples

Assessment of a panel of proliferation associated genes revealed consistent down-regulation in second biopsy in letrozole-treated tumours. No consistent trend in proliferation genes in non-treated tumours was observed.

Figure 8-1. No consistent changes in proliferation genes in non-treated samples Heatmap of proliferation associated genes in letrozole-treated and non-treated samples. Samples are ordered left to right by interval from core biopsy to surgery (days), in letrozole treated and non-treated cohorts. Colours relative to pre-treatment sample. Green – lower expression, red – higher expression